THE IMPACT OF SOCIAL STRESS ON ACUTE THEILER'S MURINE ENCEPHALITIS VIRUS INFECTION

A Thesis

by

ROBIN RANEE JOHNSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2003

Major Subject: Psychology

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Approved as to style and content by:

Mary W. Meagher (Chair of Committee)	C.J.R. Welsh (Member)
Jim Grau (Member)	W. Steve Rholes (Head of Department)

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ABSTRACT

The Impact of Social Stress on Acute Theiler's Murine Encephalitis Virus Infection.

(May 2003)

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Chair of Advisory Committee: Dr. Mary W. Meagher

Stress is known to alter immune function, both in positive and negative ways. The disparate effects of stress on immune function remains an active area of investigation. This thesis investigates how the application of social disruption stress either prior to or concurrent with infection alters the neuropathogenesis of Theiler's murine encephalitis virus. Experiment 1 verified that social disruption prior to infection exacerbated the course of infection. Experiment 2 examined application of social disruption concurrent with infection, and found that this may produce a delay in symptom onset, and possibly a protective effect. Experiment 3 directly compared the two schedules to each other. The previous findings were replicated and expanded with additional measures (both behavioral and physiological) that further verified the earlier findings. Social disruption applied prior to infection resulted in greater behavioral and physiological exacerbation of the disease. Concurrently applied stress remained protective or inhibitory in the disease progression. Timing of stress is one of several quantitative aspects of stress that has been found to impact the stress-immune interaction and should be further investigated.

ACKNOWLEDGEMENTS

Thank you to all who contributed to the completion of this thesis. A brief list must include my advisor, Dr. Mary Meagher, for patience and brilliance, Drs. Jane Welsh, Tom Welsh and Ralph Storts for much of the same patience and brilliance. My undergraduate helpers were essential and my gratitude immeasurable; the list is long, but a few stood out: Larissa Husband, Gabby Oroza, Heath McCullough, and Patrick Bridegam- you guys are the best! Heath and Patrick were also wonderful friends who helped keep me as sane as is possible. My Mom and Dad, thanks for being my parents, and all the support- honest Mom, I really couldn't have done better than God did! And finally thanks to my best friend, Frankie Lynn Martinez- she is my inspiration, my sanity, and no one could ask for more than a friend who lets you come and write in her home in the mountains of Colorado. Thank you all.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Stress has been implicated as an important factor in the course of neurodegenerative diseases (Busciglio et al., 1998; Rosch, 1979). Neuroinflammation is thought to be the major mediator of neurodegenerative diseases, both as an underlying cause and as an exacerbating factor (Floyd, 1999; McGeer and McGeer, 1995). In addition, stress can induce central nervous system (CNS) inflammatory processes leading to neuroinflammation (Cirulli et al., 1998; Esposito et al., 2001; Maes, 2001). Due to this relationship between neurodegeneration, stress and inflammation, it is important to understand how these factors interact. Therefore, the purpose of this thesis is to examine the role that social stress plays in the development of CNS inflammation using Theiler's murine encephalitis virus (Theiler's virus) infection as a model for CNS inflammation.

CNS inflammation is mediated mainly through resident immune cells such as microglia and astrocytes (Gonzalez-Scarano and Baltuch, 1999; McGeer and McGeer, 1995; Nguyen et al., 2002; Zheng et al., 2000). Once the resident cells are activated, a subsequent increase in blood-brain barrier permeability occurs. The leaky blood-brain barrier allows peripheral innate (i.e. macrophages) and adaptive (i.e. T-cells) immune processes to infiltrate to the CNS (Lassmann, 1991). These cells can then increase and perpetuate CNS inflammation (Andersson et al., 1992; de Vries et al., 1997; Lassmann et al., 1991; Matyszak and Perry, 1995; Matyszak et al., 1997).

Amazingly, recent studies have demonstrated that stress alone can directly influence CNS inflammatory processes. Both blood-brain barrier permeability and proinflammatory cytokine levels (in the CNS) are increased due to stress in both human and animal studies. For example, stress has been

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shown to increase blood-brain barrier permeability in Multiple Sclerosis (MS) patients (Mohr et al., 2000). In addition, many studies have found that IL-1 and IL-6 are elevated due to stress in humans and rodents (Maes et al., 1998; Paik et al., 2000; Stark et al., 2002). Finally, multiple studies have shown that elevated proinflammatory cytokines (Il-1, IL-6, and TNFα) increase blood-brain barrier permeability in rodent stress models such as restraint, foot shock, and open field (reviewed in Maes, 2001).

Stress is also known to impact many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and MS. However, these findings have not been conclusive. Human studies have shown stress to be both detrimental and favorable. For example, several studies have shown that stress increases the risk of experiencing a clinical onset and relapse in MS (Ackerman et al., 2000; Franklin et al., 1988; Grant et al., 1989; Sibley, 1997; Warren et al., 1982; Warren et al., 1991). In addition, Alzheimer's symptoms (Motomura et al., 1998) and Parkinson's disease symptoms (Schwab and Zieper, 1965) are exacerbated following periods of elevated stress. In contrast, Nisipeanu and colleagues (1993), demonstrated that fewer reports of symptom onset and/or relapse occurred in MS patients following the bombings of Tel Aviv during Desert Storm (1992-1993), indicating that the stress may have reduced symptoms.

Some studies suggest that the effects of stress on disease course depend on the nature of the stressor. For example, clinical exacerbation of MS symptoms were related to marital and job-related stress but not to major life stressors (Sibley, 1997). Recently, Mohr and colleagues (2000) demonstrated that certain types of stress (conflict, disruption, daily hassles, but notably not major life stressors) predicted later development of new brain lesions in MS patients. However, none of the stressors examined by Mohr and colleagues altered symptom reports. Additionally, Ackerman and colleagues (1996, 1998) were not able to find immune response differences between MS patients and

controls following acute laboratory stressors. Taken together, these studies suggest that severity, quality, and chronicity of stressors differentially effect disease course.

Many recent animal model studies have also demonstrated that qualitative and quantitative aspects of stress differentially impact immune function. Qualitatively, acute and chronic stress are known to impact humoral and cellular immune function in a divergent manner (Dhabhar and McEwen, 1996; Dhabhar and McEwen, 1997; Dhabhar and McEwen, 1999). In addition, when restraint stress was applied in relation to sensitization altered delayed type hypersensitivity responses (Flint et al., 2001). Other quantitative aspects that impact immune function include both strain (in rodents) and gender (Kerr et al., 1996; Palanza et al., 2001). In addition qualitative characteristics of stress also alter immune outcomes. For example, social disruption stress has been shown to diverge from restraint stress on immune measures such as glucocorticoid resistance and the release of pro-inflammatory cytokines (Quan et al., 2001).

Although it is clear that stress impacts inflammatory neurodegenerative diseases such as MS, the role that it plays is complex, and relatively little is known about the mechanisms that mediate this process. The neuroimmune and neuroendocrine pathways associated with stress and immune responses that may explain the divergent outcomes on disease course remain unexplored. Because human studies are unlikely to resolve these issues, animal studies are needed to determine the mechanisms mediating the divergent effects of stress on disease course. Theiler's virus infection in mice has been used for many decades to study MS. Using this model, the many possible aspects of stress-immune interactions can be explored in a controlled manner.

Theiler's virus infection provides a well characterized model to study the pathogenesis of CNS inflammation and therefore it is an excellent model for examining the interaction between stress and CNS inflammation (Aubert et al., 1987; Fujinami and Zurbriggen, 1989; Welsh et al., 1990). It is a picornavirus

that causes an asymptomatic gastrointestinal infection associated with encephalitis and paresis in the early phase of infection (Theiler, 1934). If the virus persists in the CNS, the infection will result in chronic demyelination in susceptible strains of mice such as BALB/cJ (Lipton, 1975).

Our laboratory has previously examined the impact of chronic restraint stress on the course of Theiler's virus infection (Campbell et al., 2001). These studies indicated that restraint stress administered beginning the same day as inoculation led to a decrease in inflammation compared to controls. This decrease in inflammation was associated with an increase in behavioral manifestations of encephalitis, higher mortality, and higher viral titers.

In order to examine both the generalization of these restraint stress findings, as well as to begin to examine how dissimilar stressors may account for divergent immune outcomes found in much of the human literature, a series of studies using social disruption stress were conducted. Social disruption is a model of social stress that has been developed to study immune modulation (Avitsur et al., 2002; Avitsur et al., 2001; Quan et al., 2001; Stark et al., 2002; Stark et al., 2001). Social disruption has been shown to differ greatly from restraint stress, resulting in greater inflammation and mortality (Quan et al., 2001). One possible mechanism underlying the differences in inflammation found between social disruption and restraint is glucocorticoid resistance. Glucocorticoid resistance is induced by social disruption (elevated inflammation) but not restraint (lower inflammation). It occurs when immune cells, such as macrophages and lymphocytes that are normally down regulated by glucocorticoids begin to resist this regulation, leading to an excessive immune response and increased likelihood of autoimmune problems. Glucocorticoid resistance is also known to develop in many patients with chronic immune conditions, such rheumatoid arthritis and MS (DeRijk and Sternberg, 1997).

The current studies examined the effects of social disruption stress on Theiler's virus infection. Experiment 1 followed the timeline of the social

disruption developers (Avitsur et al., 2001; Quan et al., 2001; Stark et al., 2001), with administration of stress for one week prior to immune challenge. This study demonstrated that social disruption was effective in altering the Behavioral and physiological manifestations of Theiler's virus infection. A follow-up study with a timeline similar to the previous restraint stress study (Campbell et al., 2001) was conducted. Experiment 2 administered social disruption for one session prior to infection, and remaining sessions followed after inoculation with Theiler's virus. The results from Experiment 2 contrasted with those of Experiment 1, and thus Experiment 3 was conducted to directly compare the two timelines and delve further into how social disruption alters the Behavioral and physiological manifestations of Theiler's virus infection.

GENERAL METHODS

Subjects

Male BALB/cJ mice were used in all experiments (either from our breeding colony, or supplied by Jackson Labs, Bar Harbor, ME). Mice were housed three per cage in standard Plexiglas cages, with ad libitum access to food and water, with the exception of the two-hour social disruption sessions. The age of the mice varied based on the timeline used in each experiment, however all mice were allowed to acclimate to the experimental cage conditions at least one week prior to the stress manipulation.

Procedures

Social Disruption

Dominants (sexually experienced males, 6-8 mo of age or greater) were introduced into the cage of resident mice in the social disruption condition at dark cycle onset. Dominants remained in the residents' home cage for two hr each session (5 p.m.- 7 p.m.), for six sessions over a seven day period. Social disruption occurred in a procedure room, separate from the homeroom where animals were normally housed. Home cage controls remained in the homeroom for the duration of the social disruption. Dominants that did not attack within ten min of session initiation were replaced, and the session continued for the remaining two hrs. All social disruption sessions were videotaped through infrared cameras.

Dominant Selection

Dominants were selected based on latency to attack both peers and adolescents. The prospective dominants were first placed into a new cage with another prospective dominant. Latency to attack was record, and the animal that attacked first was then tested against adolescents. The prospective dominant was placed into the homecage of adolescent mice that were not used for any other purpose in the studies. Latency to attack one of the resident mice

was recorded. Dominants attacked peers within thirty seconds and adolescents within two min consistently on three separate occasions.

Bleeding

Saphenous leg bleeds were conducted within two hrs post-stress of the third stress session. Approximately 50 μl of blood was collected in EDTA coated capillary tubes, and centrifuged plasma was then frozen at -80°C until the time of radioimmunoassay.

Corticosterone Levels

Plasma concentrations of corticosterone were determined with radioimmunoassay (RIA kit from ICN Biomedical, Inc., Costa Mesa, CA).

Infection

Isoflurane (IsoSol from Vedco, # 58-1825-2/R2) anesthetized mice were inoculated with 5 X 10^4 p.f.u. of either the BeAn strain of Theiler's virus in a 20 μl volume of PBS or PBS alone intracranially in the right parietal lobe. Inoculation for all subjects occurred at 9 p.m. (two hr following the end of social disruption sessions), at post-natal day (pnd) 35.

Materials

Infrared Monitors

The cameras used to monitor and record social disruption sessions were Lorex CVM6790P model infrared cameras.

Behavioral Measures

Hindlimb Impairment Assessment

Blind rating of paresis/paralysis was conducted every 2-3 days post infection (p.i.). This score was based on Hindlimb weakness, possible paralysis, ability to cling and locomotion while inverted on a grid above the home cage. The scale ranged from 0 (no impairment) to 5 (complete paralysis) (detailed procedure may be found in Appendix 1). Each Hindlimb was assessed independently, and reported as a combined score. This scale had inter-rater reliability of r = .9. Wounding was also assessed during these sessions.

Body Weights and Food Intake

Body weights and food intake per cage were assessed regularly throughout all experiments as a measure of illness Behavior.

Physiological Measures

Glucocorticoid Resistance and Cell Proliferation

Spleens were harvested aseptically, weighed and immediately placed in 4 mls ice cold HBSS (Hank's balanced salt solution, Sigma #H6393) and mashed with forceps to form a single cell suspension. The solution was then filtered through 250 μm mesh to remove debris. The cell suspension was then centrifuged (2000 rpm/20°C/5 min) and the re-suspended pellet treated with lysis buffer (Sigma #R7757) to remove red blood cells, followed by a wash of HBSS + 10% FBS (heat inactivated (H/I), Equitech-Bio, Inc.). Cells were then centrifuged once again (2000 rpm/20°C/5 min), and the pellet resuspended in 10 mls of HBSS + 10% FBS (H/I). Viable cells were then counted using trypan blue dye exclusion and re-suspended at 2.5 X 10^6 cells/ml in supplemented RPMI (Sigma #R0883) +10% FBS (H/I) (supplementation: .75% sodium bicarbonate, 10 mM Hepes buffer (Sigma # H3784), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (Gibco penicillin-streptomycin, #15140-122), 1.5 mM l-glutamine (GibcoBRL # 25030-081), and .00035% 2-mercaptoethanol (Sigma #M7522)). Lipopolysaccharide (Sigma # L6529) was added at a concentration of 1 μg/mg for mitogen stimulation. Glucocorticoid resistance was tested by exposing aliquots of each suspension to dilutions of corticosteroid (0-5 μM, Sigma #C2505) dissolved in 2% ethanol in supplemented RPMI. Cell suspensions were placed in triplicate in flat-bottomed 96-well micro-titer plates in 100 μL aliquots and incubated for 48-72 hrs at 37°C and 5% CO_2. After incubation, the cell proliferation assay was performed.

Cell proliferation was assessed with the CellTiter 96 Aqueous non-radioactive proliferation assay kit from Promega (#G5421,Madison, WI). The tetrazolium substrate solution was prepared according to the instructions and 20

μL was added to each well of the 96 well plates. Living cells convert the substrate to formazyn, forming a brown precipitate. The plates were incubated for three hr for the color changes to develop. Color changes were quantified by optical density readings at 490 nm from an EMAX ELISA plate reader (Molecular Devices). Mean optical density values were used, with the RPMI+ 10% FBS (H/I) well values subtracted from each of the corresponding lipopolysaccharide stimulated values.

Sacrifice

All mice were sacrificed using 0.2 ml of 50 mg/kg pentobarbital in all experiments. The following organs were dissected and weighed: spleen, thymus, adrenal glands, spinal cord, and brain. Additional organs specific to Experiment 3 included testicles, epididymis, and seminal vescicles.

Tissue Processing

For viral clearance assays, tissue was flash frozen in liquid nitrogen and then transferred to a -80°C freezer until the time of assay.

For histology, the animals were gravity perfused with 10 mls of PBS followed by 10 mls of 10% formalin. The brain and spine were then left intact and allowed to fix overnight. The following day, the brain was removed from the skull and sectioned coronally into four samples. The first cut was made through the cerebral hemispheres at the junction between the rostral one-third and the caudal two-thirds of the hemispheres. The second cut was made at the apex of the longitudinal sulcus. The final cut was made just rostral to the cerebellum.

The vertebral column was cleared of excess tissue and sectioned into 12-2 mm slices. These slices were ideally: four cervical, four thoracic, and four lumbar sections.

All histological tissues were dehydrated and embedded in paraffin. Five micron slices of each block (one brain with four sections, one spinal cord with 12 sections) were routinely stained with hemotoxylin and eosin (H&E) for microscopic examination.

Viral Clearance

Frozen tissues (brain and spinal cord) were homogenized with 2-.5 ml aliquots of DME (Gibco #10569-010) + 10% FBS, sonicated (2 X 10 s) and centrifuged (2000rpm/20°C/5 min). The homogenate was then frozen again until the time of assay. At the time of the assay, tissue was thawed and centrifuged once again (2000 rpm/20°C/5 min). Viral titers in brain and spinal cord were measured by plaque assays on L2 cells. Dilutions of 1:1, 1:10, 1:100, and 1:1000 of homogenate were tested for all samples to gain the most accurate assessment of viral load. Assays were scored based on the number of plaques formed on the L2 cells and calculated per gram of tissue harvested.

Histology Scoring

Lesions of both the brain and spinal cord were scored in a similar manner, and a rater made all assessments blind to condition. Lesion types include perivascular cuffing (macrophage accumulation around vascular vesicles), meningitis (microglia and macrophages accumulating in the meninges), and microgliosis (increased microglia cellularity). In the spinal cord, peripheral nerves were also assessed for degeneration (perforations formed from demyelination).

Statistics

Analysis of variance (ANOVA) was used to evaluate differences across conditions. In the case of measures that were repeated over time, a repeated ANOVA was used. Viral titers required a log transformation, however all other data remained untransformed. Further assessment of group differences was assessed by Duncan's post hoc analysis.

EXPERIMENT 1

Previous studies in our laboratory demonstrated that restraint stress suppressed CNS inflammation in mice infected with Theiler's virus, leading to increased mortality. Interestingly, some studies indicate that social stressors in rodents can be more detrimental than restraint stress. For example, Quan and colleagues (2001) found that social disruption led to increased inflammation and mortality in animals exposed to an endotoxin challenge compared to restraint stressed animals. The purpose of Experiment 1 was to establish the effects of social disruption on the course of Theiler's virus infection.

The effects of social disruption on inflammation are thought to be due to glucocorticoid resistance. Social disruption has been shown to induce glucocorticoid resistance, whereas restraint stress fails to do so (Avitsur et al., 2001; Quan et al., 2001; Stark et al., 2001). Glucocorticoids regulate the inflammatory process (Sternberg, 2001), so glucocorticoid resistance may mediate the increased inflammation observed in socially disrupted animals.

In the original studies, social disruption was applied for the week prior to an immune challenge (Quan et al., 2001). Therefore, Experiment 1 examined the impact of social disruption stress administered one week prior to inoculation with Theiler's virus. In order to gain the most complete picture of the impact of social disruption on Theiler's virus infection, multiple Behavioral and physiological measures were required. Therefore Experiment 1a examined behavioral manifestations and CNS viral load. Experiment 1b followed to examine the histological changes associated with the behavioral manifestations of Theiler's virus infection. As the pattern of behavioral outcomes was similar across both 1a and 1b, only the histological data are presented from 1b. It is hypothesized that social disruption will negatively impact the course of Theiler's virus infection. This could occur either by suppression of inflammation as was found with restraint stress (Campbell et al., 2001), or by excessive inflammation found with social disruption stress (Quan et al., 2001).

Methods

Subjects

In Experiment 1a, the adolescent subjects were bred in our animal colony, and weaned at pnd 21, n = 48. Subjects were placed in cages of three, with non-littermates when possible, and randomly assigned to stress and kill date condition at the time of weaning. Residents were then allowed to acclimate for one week while baseline measures were acquired. All results, with the exception of histology are exclusively from Experiment 1a, as 1b had a similar pattern across measures.

In Experiment 1b, the resident mice were acquired from Jackson Labs at pnd 19 , n = 16. Upon arrival, the animals were weighed and assigned to a home cage in a counter balanced manner based on weight. Cages were then randomly assigned to a stress condition. Mice were allowed to acclimate for one week while baseline measures were taken. Figures 1 and 2 show the experimental design and timeline respectively.

A total of 64 animals were used in Experiment 1.

Social Disruption	**Infection**	**Sacrifice**
SDR	Infected	Day 7 p.i.
		Day 21 p.i.
	NON-Infected	Day 7 p.i.
		Day 21 p.i.
NON-SDR	Infected	Day 7 p.i.
		Day 21 p.i.
	NON-Infected	Day 7 p.i.
		Day 21 p.i.

Figure 1. Design- Experiment 1.

PND/Day p.i.	PND 21	PND 28-35	PND 35/ 0 p.i.	PND 42/ 7 p.i.	PND 63/ 28 p.i.
Event	Wean/ Arrive	SDR	Infection	Sacrifice- Viral Clearance	Sacrifice- Viral Clearance and Histology

Figure 2. Timeline- Experiment 1.

Procedures

Social Disruption. Resident mice were administered social disruption stress beginning at pnd 28, one week prior to infection (Figures 1& 2). Dominants were selected from the breeders in the colony.

Infection. Half of the subjects were inoculated with virus, while the other half were mock infected.

Sacrifice. Animals were sacrificed at day 7 p.i./ post stress (p.s.) or day 28 p.i./ p.s. in order to asses both the initial infection and the course of the early phase of infection.

Results

Figure 3 depicts mean hindlimb impairment scores across days p.i. as a function of infection and social disruption stress. As expected, infection resulted in hindlimb impairment; and social disruption further exacerbated the level of impairment over time. An ANOVA confirmed a significant main effect for infection, F (1,20) = 116.129, p < .001, and social disruption, F (1,20) = 18.212, p < .001, as well as a significant interaction between Infection X Social Disruption, F (1, 20) = 19.546, p < .001. A significant three-way interaction between Infection X Social Disruption X Day P.I., F (8,160) = 3.742, p < .001, indicated that the effect of social disruption and infection on hindlimb became worse with time. Post hoc means comparison revealed that the infected/ socially

disrupted group exhibited greater impairment than the infected/non-socially disrupted group at day 11 p.i., and through the completion of the experiment.

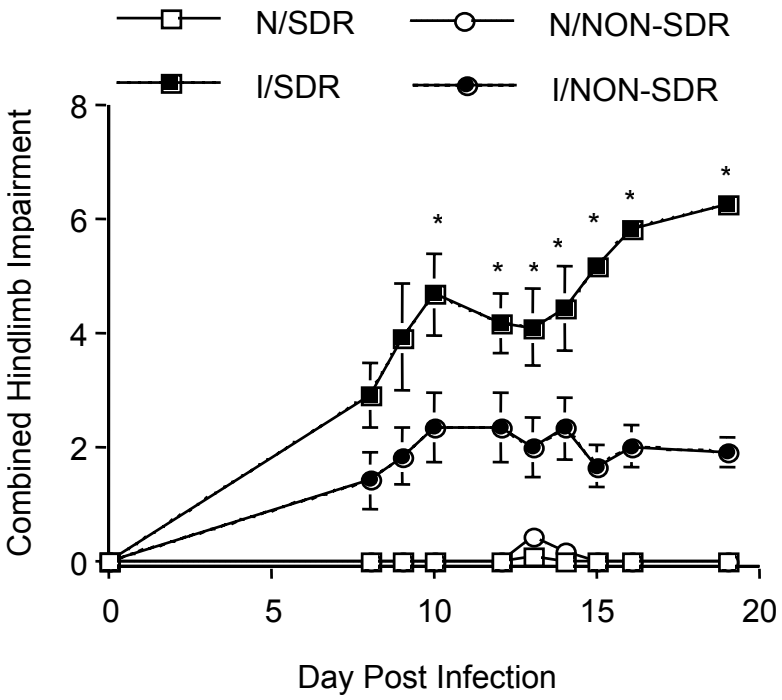

Figure 3. Hindlimb Impairment- Experiment 1.
Effects of infection (I) and social disruption (SDR) on mean hindlimb impairment +/- SEM. Mice underwent social disruption for one week prior to infection (days –7 through 0 p.i.). Assessment began at day 8 p.i.

Figure 4 presents mean body weight change from baseline scores. Infection is often associated with weight loss, however the adolescents used in these experiments had not yet reached normal weights. Therefore, the failure to gain weight at a normal rate (i.e. the change from baseline weights), is a more accurate assessment of illness Behavior. It is apparent that infection reduced normal weight gain. In addition, social disruption altered normal weight gain as

a function of infection. Social disruption reduced weight gain even further in the infected animals, but in the non-infected animals, social disruption increased weight gain over time. An ANOVA confirmed a significant main effect of infection, F (1, 20) = 16.835, p < .001, as well as a significant Infection X Day P.I. interaction, F (8, 160) = 17.741, p < .0001. Finally, a significant three-way interaction between Infection X Social Disruption X Day P.I. interaction, F (8, 160) = 2.214, p < .05, was revealed. No other differences were significant, p > .05. No significant differences were obtained for food intake, but this may reflect the small number observations per condition, (n = 3 cages).

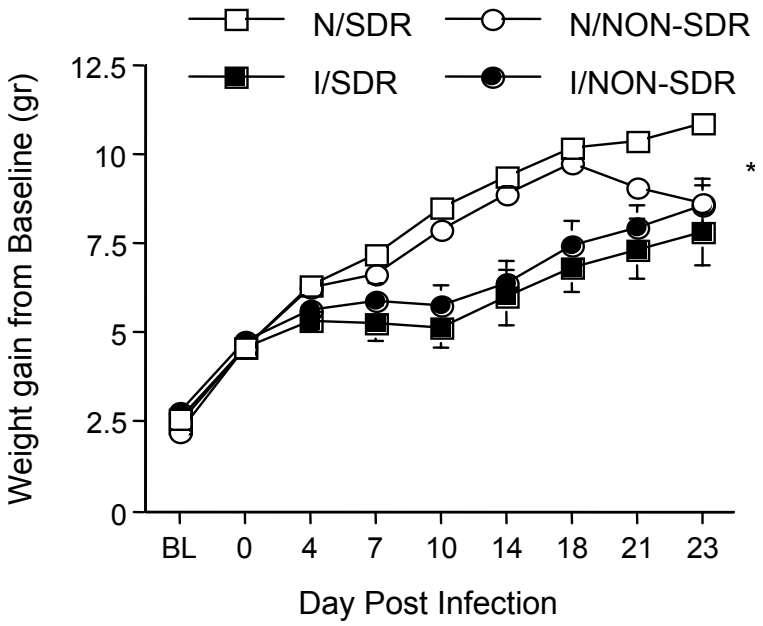

Figure 4. Body Weight Gain- Experiment 1.
Compares mean body weight gain +/- SEM across time as a function of infection (I) and social disruption (SDR). Social disruption occurred between BL and 0 p.i.

Social disruption was associated with higher CNS viral titers at day 7 p.i. (Figure 5). ANOVA revealed a significant main effect for social disruption on total viral load (virus in both brain and spinal cord tissue), F (1, 10) = 3.728, p < .01. Although there appears to be a trend toward increased viral load in the spinal cord, this difference was not statistically significant, F (1, 10) = 2.526, p =.08 (data not shown). Finally, only two animals in the social disruption/infection group had detectable viral loads at day 28 p.i., these animals did not differ on any other measure from the others in this condition. The infected/ non-socially disrupted group had no detectable viral load. This small difference was not statistically significant, p > .05.

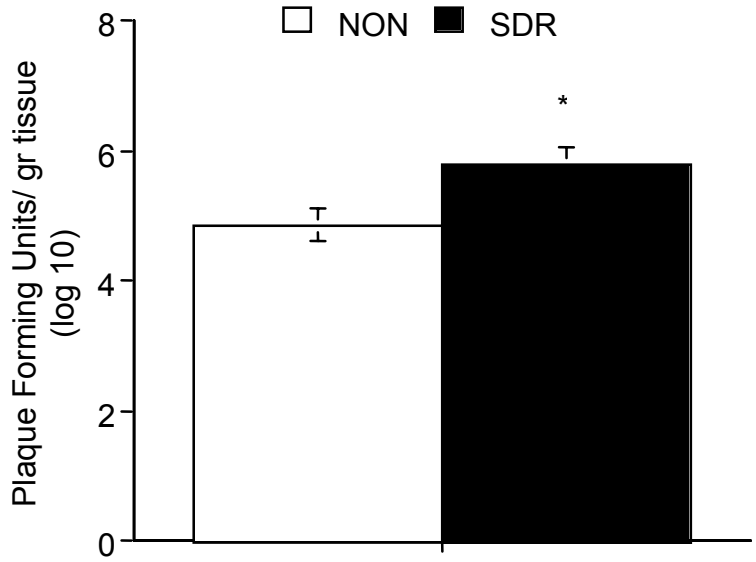

Figure 5. Viral Load- Experiment 1.
Mean total viral load +/- SEM of infected animals at day 7 p.i., as a function of social disruption (SDR). Only infected animals are shown, as no virus was found in the non-infected conditions.

Figure 6 depicts the cell viability of the glucocorticoid resistance assay. Glucocorticoid resistance was determined by slope analysis. Resistant cells will continue to function in the presence of increasing levels of corticosteroids, thus the slope if these lines should be near zero. Only the non-infected socially disrupted animals developed resistance. The average slope for these animals was -.018. ANOVA revealed a main effect for infection, $\underline{F}$ (1, 18) = 5.818, $\underline{p}$ < .05, and a marginal effect for social disruption, $\underline{F}$ (1, 18) = 3.439, $\underline{p}$ = .08. The interaction was not significant, however means comparisons indicate that the non-infected socially disrupted animals differ significantly on slope from all other groups.

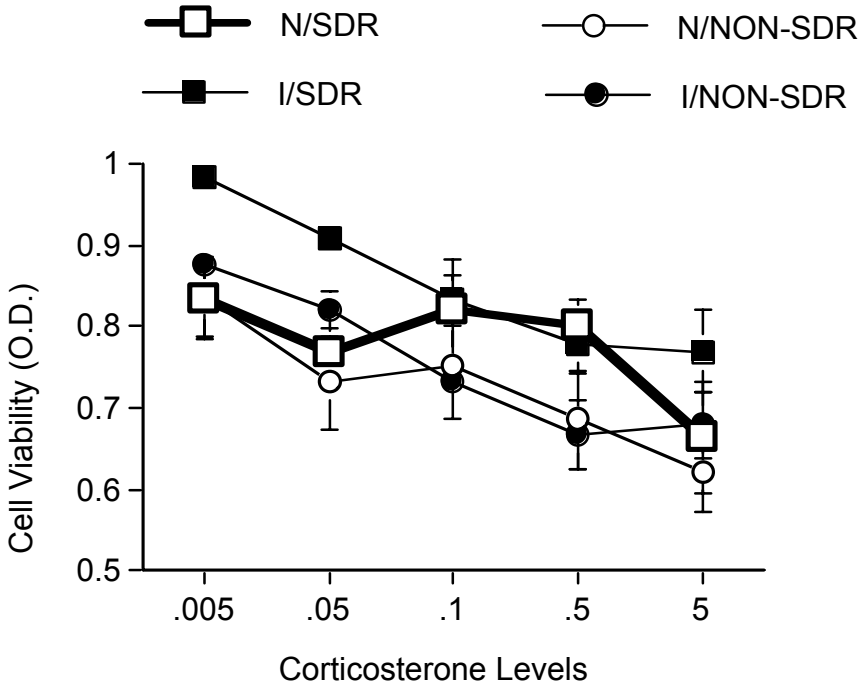

Figure 6. Glucocorticoid Resistance- Experiment 1.
Effect of increasing corticosteroid levels on mean proliferation/ viability (O.D.) of splenocytes +/- SEM as a function of infection (I) and social disruption (SDR). The SDR conditions underwent six social disruption sessions prior to infection. Spleens were harvested at 7 p.i.

Figure 7 depicts the corticosteroid levels within two hours of social disruption. Social disruption resulted in a two-fold increase in corticosteroid levels. An ANOVA revealed a significant main effect for social disruption, F (1, 19) = 17.598, $p < .0001$.

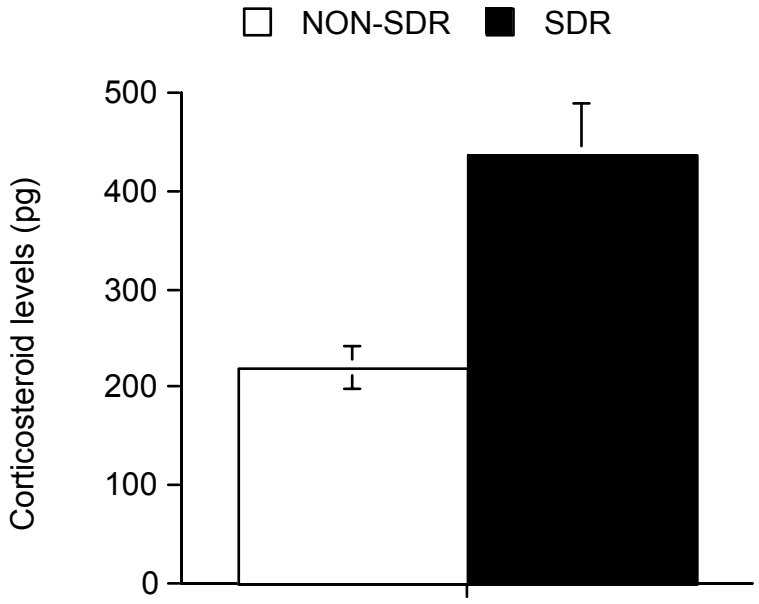

Figure 7. Corticosteroid Levels- Experiment 1.
Comparison of mean corticosteroid levels, +/- SEM as a function of social disruption (SDR). Samples were taken immediately following the third social disruption session. The animals were not infected at the time of sample collection for the corticosteroid assay.

Figure 8 shows adrenal weights at days 7 p.i. and 28 p.i. Infection reduced adrenal weights in this model, while social disruption in the absence of infection increased adrenal weights. Social disruption alone was associated with a two-fold increase in adrenal weight, while in combination with infection this increase was not evident. An ANOVA verified a main effect for social disruption,

F (1, 10) = .00024, p < .05, and infection F (1, 10) = .00028, p < .05. In addition, ANOVA revealed a significant interaction of Infection X Social Disruption, F (1, 20) = .000216, p < .05. Post hoc mean comparisons indicated that social disruption reduced adrenal weight at day 7 p.i., but increased adrenal weight at day 28 p.i. Infection alone also reduced adrenal weights at both days p.i., but when infection was combined with social disruption no changes occurred.

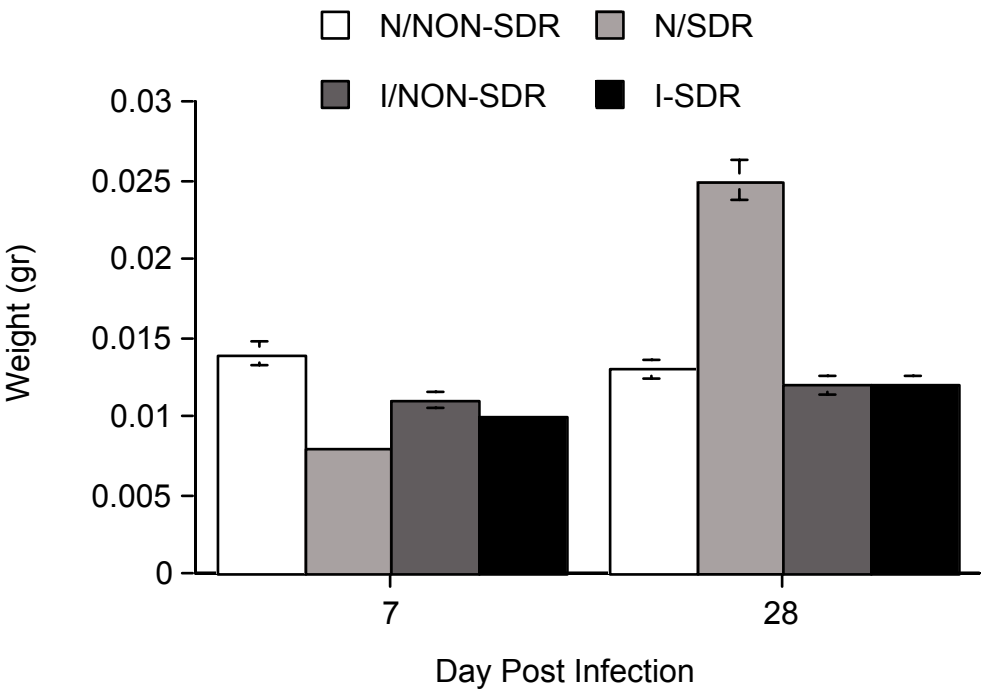

Figure 8. Adrenal Weights- Experiment 1.
Effect of infection (I) and social disruption (SDR) on mean adrenal weights (gr) +/- SEM. Adrenal glands were harvested at sacrifice either day 7 p.i. or day 28 p.i. All social disruption condition animals underwent social disruption for one week prior to infection.

Histological analyses of the spinal cord are presented in Figure 9. Overall, a pattern of elevated inflammation (indicated by microgliosis, meningitis, and

perivascular cuffing) developed in the infected/social disruption group compared to the infected/non-stressed group, however the differences are not significant (p > .05).

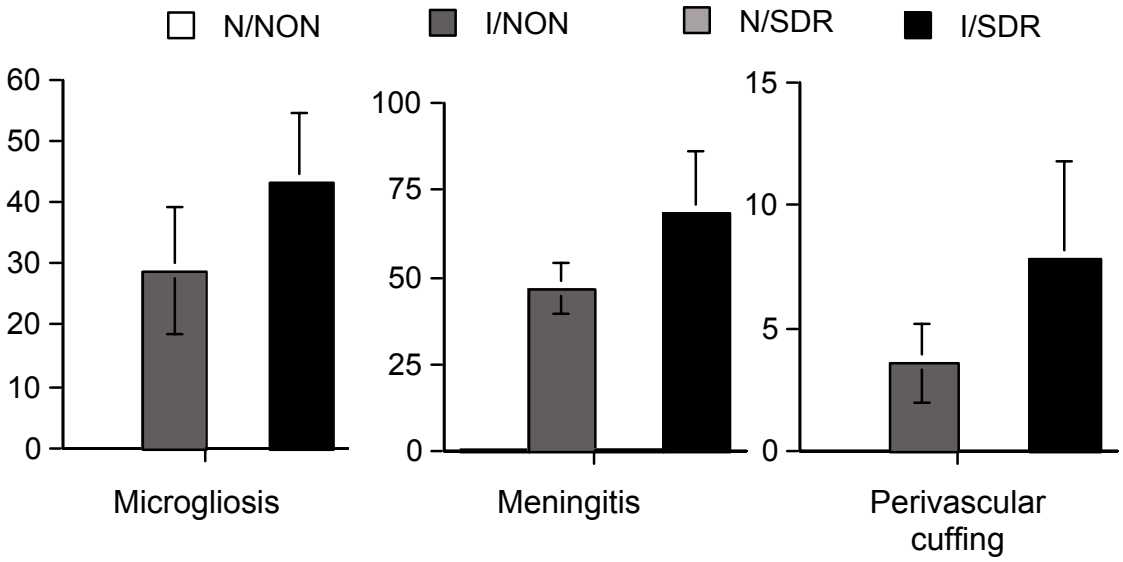

Figure 9. Spinal Cord Histology- Experiment 1.
Effect of Social Disruption (SDR) on histological inflammation (microgliosis, meningitis, and perivascular cuffing) within the spinal cord. Tissue was harvested at day 28 p.i.

Discussion

The present study demonstrated that the application of social disruption prior to infection resulted in a more severe disease course during early Theiler's virus infection. Social disruption led to significantly greater hindlimb impairment. Physiologically, viral loads and inflammation were both slightly (although not

always significantly) elevated. Taken together, these data suggest that social disruption negatively impacted the course of Theiler's virus infection.

These findings are in general agreement with those found by others using social disruption (Quan et al., 2001), having demonstrated increased inflammation and disease exacerbation. However, glucocorticoid resistance, the mechanism proposed by those studies for elevated inflammation, developed only in the non-infected animals exposed to social disruption. In addition, adrenal weights (a measure of corticosteroid activity) were only altered in the non-infected animals exposed to social disruption as well. Finally, both the infected and non-infected animals exposed to social disruption had normal elevations of corticosterone associated with the stressor. Taken together these findings indicate that social disruption alone may alter corticosteroid function, but that Theiler's virus infection may suppress this action.

EXPERIMENT 2

As noted previously, our laboratory has demonstrated that restraint stress increased behavioral signs and mortality in acute Theiler's virus infection, while decreasing inflammation. In Experiment 1, social disruption applied prior to infection with Theiler's virus led to increased inflammation and viral loads associated with elevated Behavioral signs of disease. These contrasting outcomes are confused by the fact that Campbell and colleagues (2001) administered restraint stress for one session prior to infection, and the remaining course of restraint followed after infection. In order to more accurately begin to examine how these two different stressors compare in relation to the development of Theiler's virus infection, Experiment 2 followed the Campbell and colleagues (2001) timeline, with social disruption administered concurrent with infection.

We know from studies dating back several decades (Mason, 1971) that psycho-physical stressors (such as restraint) and psycho-social stressors (such as social defeat) have resulted in divergent neuroendocrine outcomes. More recently it has been demonstrated that restraint stress and foot shock stress led to divergent cardiovascular stress responses (Adams et al., 1987). In addition, "processive" stressors were shown to utilize separate neurological stress pathways than "systemic" stressors (Herman and Cullinan, 1997). Finally, social stress studies have found that social disruption and social reorganization stress increased inflammation mortality and viral titers compared to restraint stress animals (Quan et. al., 2001; Sheridan et al., 2000). Taken as a whole, these studies indicate that matching the timeline of restraint stress used by Campbell and colleagues. al. (2001) will aid in understanding the results demonstrated in Experiment 1.

Methods

Subjects

Mice were acquired from Jackson Labs and arrived at pnd 25. Mice were then weighed and sorted in a counter balanced manner based on weight. Cages were then randomly assigned to condition, and mice were allowed to acclimate to the home cage for one week while baseline measures were taken.

Procedures

Social Disruption. Social disruption was administered beginning at pnd 35, concurrent with infection. The same dominants were used as in Experiment 1. Figures 10 and 11 outline the design and timeline of Experiment 2.

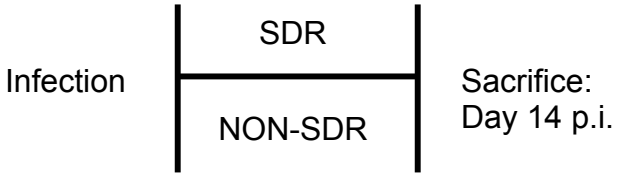

Figure 10. Design- Experiment 2.

PND/ Day p.i./ p.s.	PND 25	PND 35/ 0 p.i.	PND 42/ 7 p.i.	PND 49/ 14 p.i./ 7 p.s.
Event	Arrival	SDR Begins/ Infection	SDR Ends	Sacrifice

Figure 11. Timeline- Experiment 2.

Infection. All mice were infected with virus

Sacrifice. Mice were sacrificed at day 14 p.i. / 7 post-stress (p.s.) to examine the same point post- stress as in Experiment 1.

Results

Mean hindlimb impairment scores across day p.i. are presented in Figure 12. Once again, infection resulted in hindlimb impairment. In contrast to Experiment 1, however, social disruption applied at the time of infection suppressed hindlimb impairment. An ANOVA confirmed a significant main effect for social disruption, F (1,10) = 13.493, $p < .005$, as well as a significant infection between Social Disruption X Day P.I., F (1, 60) = 10.565, $p < .0001$. Post hoc means comparisons revealed that the socially disrupted group had significantly less impairment beginning at day 3 p.i., and remained so for the remainder of the experiment.

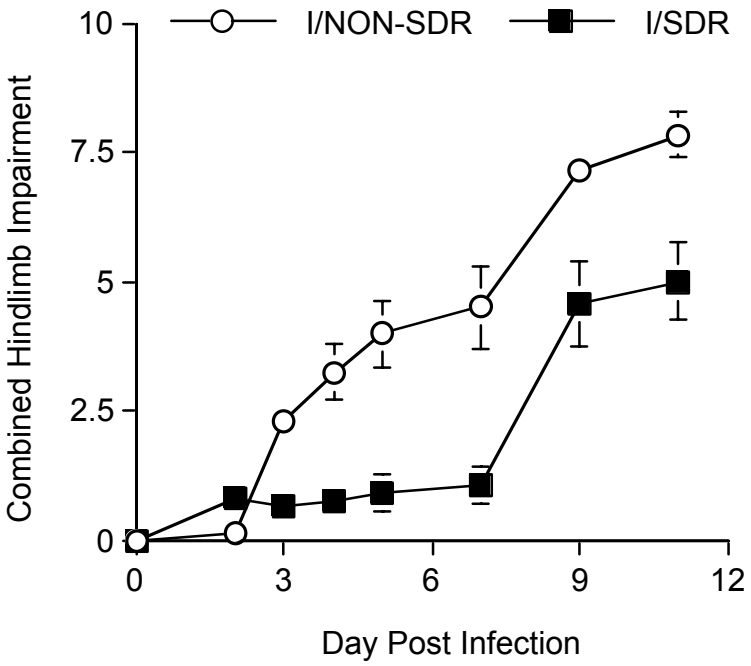

Figure 12. Hindlimb Impairment- Experiment 2.
Effect of social disruption on mean Hindlimb impairment +/- SEM. All animals were infected at pnd 35, corresponding with day 0 p.i. Social disruption (SDR) was administered for one-2 hour session that ended two hours before inoculation. The remaining five sessions were administered post-infection.

Figure 13 depicts mean body weight across days p.i. As expected, infection alone suppressed weight gain over time. However, again in contrast to Experiment 1, infection combined with social disruption increased weight gain. This was confirmed when ANOVA revealed a main effect for social disruption, F (1, 18) = 9.76, $p < .05$. Post hoc means comparisons revealed that the social disruption group had gained significantly more weight only at day 6 p.i. (note that social disruption ended at day 7 p.i.).

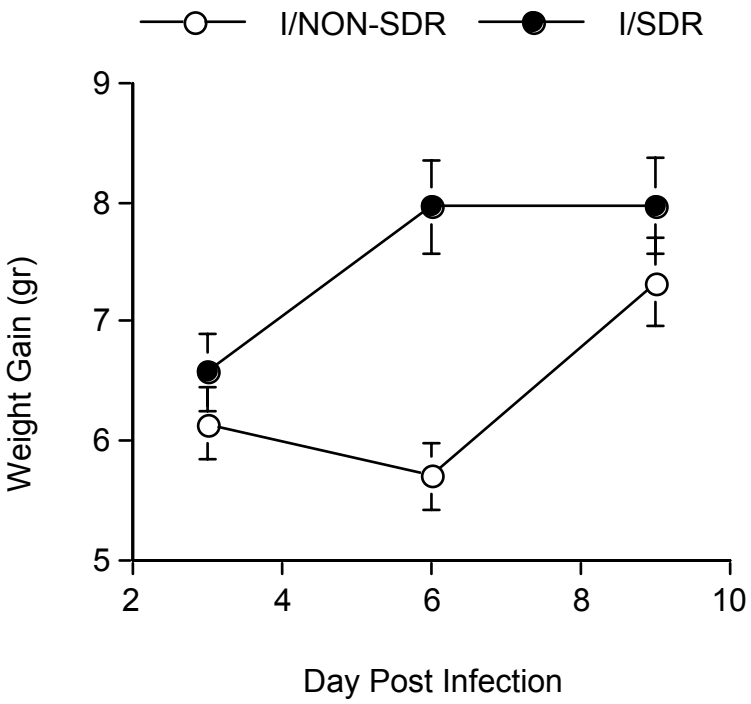

Figure 13. Body Weight Gain- Experiment 2.
Effect of social disruption (SDR) on mean body weight gain (gr) +/- SEM. Social disruption began at day 0 p.i. and ended at day 7 p.i. All animals were infected.

Glucocorticoid resistance, as in Experiment 1, did not develop. Increasing corticosterone levels were associated significant decreased cell viability, $p < .05$. Figure 14 depicts the results of this analysis.

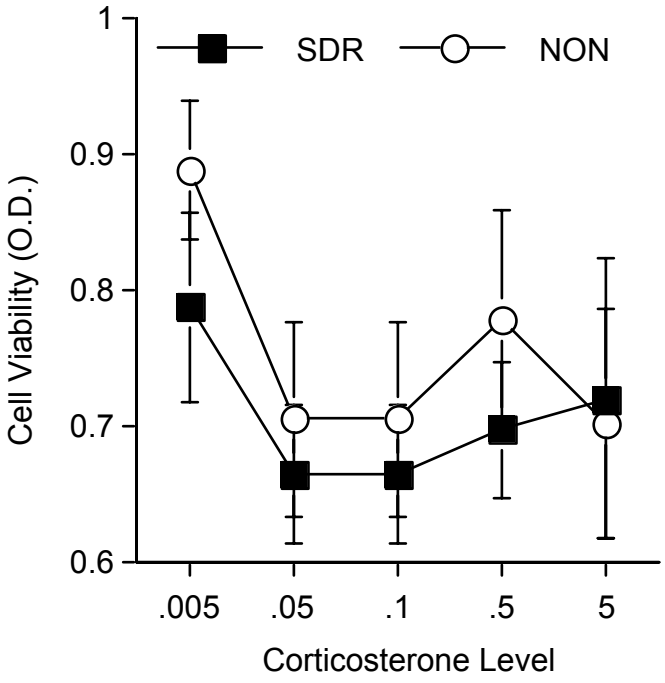

Figure 14. Glucocorticoid Resistance- Experiment 2.
Comparison of mean cell viability ratios (O.D.) +/- SEM at increasing corticosterone levels, as a measure of glucocorticoid resistance. Social disruption (SDR) animals were exposed to social disruption beginning the day of infection. No resistance developed.

Finally, the viral loads continued the pattern of divergent findings compared to Experiment 1. Figure 15 depicts the mean viral load (in plaque forming units). ANOVA revealed that the difference between the social disruption and control groups were not significant ($p < .09$), however an apparent trend toward lower levels in the concurrently stressed animals compared to controls seems to exist. It should be noted that these animals were seven days further into infection than in Experiment 1, and this alone may account for the change in pattern.

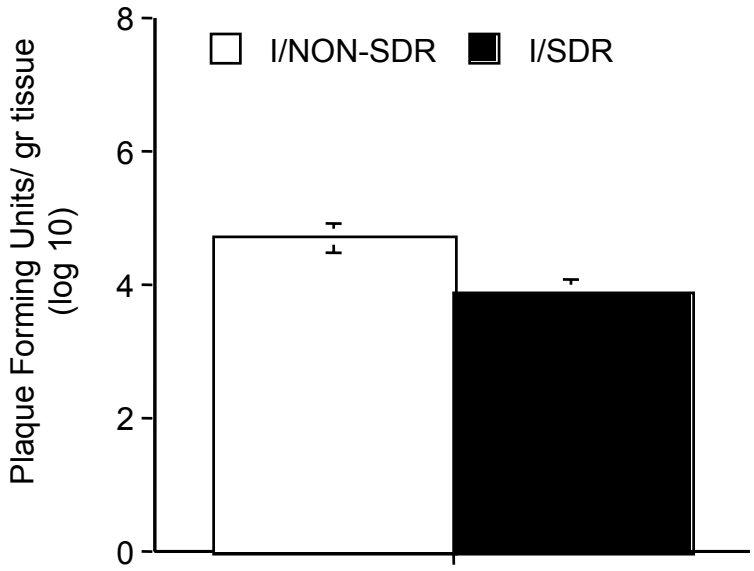

Figure 15. Viral Load- Experiment 2.
Comparison of mean viral load +/- SEM in socially disrupted (SDR) or non-stressed (NON-SDR) groups. Total viral load (combined brain and spinal cord) is shown. Tissue was harvested at day 14 p.i. All animals were infected.

Discussion

In contrast to the results of Experiment 1, the present study demonstrated that social disruption applied concurrently with infection suppressed the disease course. Behaviorally, hindlimb impairment did not develop beyond mild weakness until social disruption had ended, then impairment developed in parallel with the control animals, indicating a delay in disease onset. Body weight gain increased compared to infected controls, indicating the social disruption suppressed illness induced reductions in weight gain. In addition, viral loads were decreased in the concurrently stressed animals (at day 14 p.i.)

Taken as a whole, these findings indicate that social disruption administered concurrent to infection results in outcomes that are divergent from both restraint stress applied concurrently with infection and from social disruption applied prior to infection.

Once again, glucocorticoid resistance did not develop. Therefore it may not be a mechanism for either the positive or the negative effects of social disruption on the course of Theiler's virus infection.

EXPERIMENT 3

Experiments 1 and 2 demonstrated that the timing of stress in relation to inoculation differentially impacted the course of Theiler's virus infection. Several quantitative aspects of stress, such as chronicity (Dhabhar, 1998; Dhabhar and McEwen, 1996; Dhabhar and McEwen, 1997), and time of day (Dhabhar et al., 1994) and time of year (Bilbo et al., 2002a; Bilbo et al., 2002b) stress occurs have been explored in depth as to the effect on immune function. However, the timing of immune challenge in relation to stress is one quantitative aspect of stress that has not been greatly investigated. However, one study using restraint stress does point the importance of this issue. Flint and colleagues (2001) demonstrated that the timing of restraint stress in relation to sensitization and challenge altered delayed type hypersensitivity responses. Taken together, these findings suggest that the timing of stress in relation to immune challenge is an important determinant of immune responsiveness.

To further investigate this issue, Experiment 3 directly compares the timing schedules used in Experiments 1 and 2. This will serve two purposes. First, it will be determined if the effects observed in Experiments 1 and 2 can be replicated. Second, a definitive conclusion concerning these effects can only be made using an experimental design that directly compares both schedules. By directly comparing social disruption applied either prior to or concurrent with infection, it is possible to characterize the mechanisms mediating the divergent effects observed in Experiments 1 and 2. Additional Behavioral measures of motor impairment will be assessed in addition to the Behavioral rating scale used in the first two experiments. These include rotarod latency to fall, grid hang time, and footprint analysis. These Behavioral measures have previously been shown to be sensitive to changes in chronic Theiler's virus infection in the absence of stress (McGavern et al., 1999; McGavern et al., 2000). These measures were selected because the paresis demonstrated in the early phase of Theiler's virus infection in the BALB/cJ mice (in Experiments 1 and 2) is

similar to that observed in other strains during the chronic phase. In addition, histological and viral load data were obtained to directly compare across timing protocols. Together, the Behavioral and immunological data allowed for an in depth analysis and characterization of how social disruption impacts early Theiler's virus infection.

Methods

Subjects

Mice were acquired from Jackson Labs, and arrived at pnd 21. Mice were weighed upon arrival and assigned to cages of three based on weight in a counterbalanced fashion. Cages were then randomly assigned to stress condition and sacrifice date. These mice were run in four cycles of one cage per stress condition per sacrifice day.

Materials

Footprint Apparatus. The apparatus was composed of Plexiglas painted black. The runway was 1 m long and 12 cm wide, separated into to 2 equal halves, resulting in 2- 6 cm wide by 1m long runways. A bright white light was placed at the entrance area and a dark goal box is placed at the opposite end to promote locomotion in the correct direction.

Rotarod (Treadmill). Ugo Basile (Italy) model 7600 for five mice mouse treadmill was used for rotarod time assessment.

Procedures

Social Disruption. Animals in the pre-stress condition were administered social disruption one week prior to infection (similar to Experiment 1), beginning on day –7 p.i. / pnd 28. The concurrent stress condition animals were administered social disruption concurrently with infection, beginning at day 0 p.i./ pnd 35 (similar to Experiment 2). Dominants were retired breeders ordered from Jackson labs at least six mo of age when they arrived with the first cycle of resident mice. Dominants were housed with two sterile females in order to

increase territorial aggression. Figure 16 represents the design of Experiment 3, and Figure 17 portrays the timelines involved.

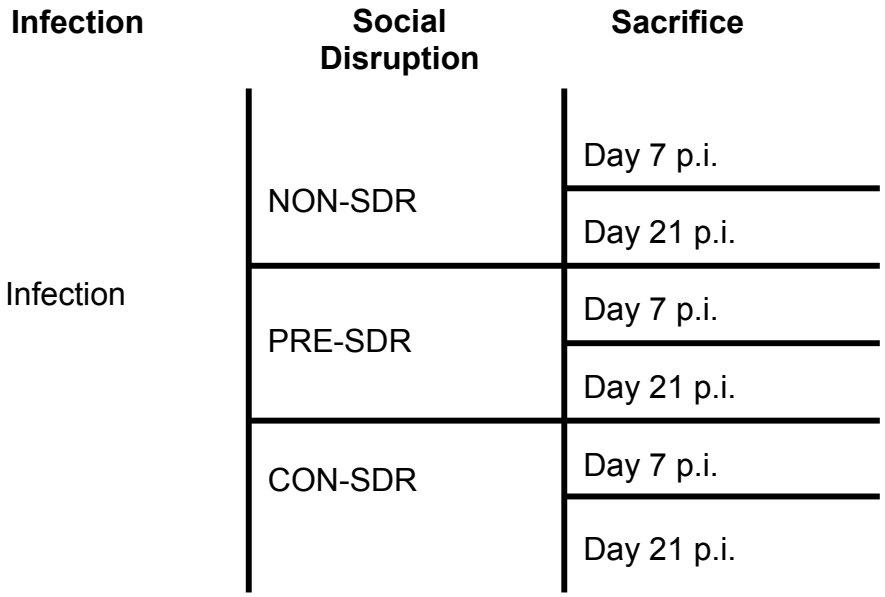

Figure 16. Design- Experiment 3.

PND/ Day p.i.	PND 21/ -14 p.i.	PND 28/ -7 p.i.	PND 35/ 0 p.i.	7 p.i.	21 p.i
Event	All mice arrive	PRE-SDR begin	PRE-SDR ends CON-SDR begins	All early sacrifice CON-SDR ends	All late sacrifice

Figure 17. Timeline- Experiment 3.

Encephalitis Assessment. Blind rating of encephalitis was conducted with the hind-limb impairment assessment every 2-3 days p.i. This score was based on the illness Behaviors of grooming, ruffling, hunching, and lethargy on a scale from 0 (no symptoms) to 4 (morbidity) (for detailed procedure see Appendix 2). Typically, BALB/cJ mice demonstrated ruffling and hunching, and thus the scores on these two sub-scales are reported. This scale has an inter-rater reliability of r = .95.

Grid Hang. Latency to fall when inverted on a standard cage floor grid was also measured. Mice were habituated to this task for three sessions prior to baseline assessment. Latency for each hindlimb to fall as well as the whole mouse to fall was recorded.

Footprint. Mice were assessed on footprint apparatus once per week, with three training sessions prior to baseline assessment. Mice were scruffed and the soles of the hindlimbs were coated with non-toxic black tempura paint. The mouse was then placed in the paper-lined apparatus and allowed to walk toward a goal box. Bright light was placed at the beginning and the goal box was dark and contained sugar pellet rewards. Footprints were scored according

to the method of McGavern, et. al. (1998, 1999), that measured the stride length and spread length for six steps, consecutive if possible. The training sessions began with each mouse being placed in the empty apparatus, then on paper, then on paper with painted feet. Each mouse received three training sessions prior to baseline assessment.

Rotarod. Mice were assessed twice per week on the rotarod apparatus. Mice were placed on the rolling barrel of a constant speed rotarod and the latency to fall was recorded. Training occurred for three sessions prior to baseline assessment. During training mice were familiarized with the apparatus and trained to maintain progressively longer periods of time, beginning with 30 s and culminating with 2 min.

Sacrifice. Sacrifice occurred at day 7 p.i. to examine the initial infection course and day 21 p.i. to examine across the course of the early phase of infection. Day 21 p.i. was chosen over day 28 p.i. (used in Experiment 1) due to the increased likelihood of having differential viral clearance and inflammatory histological lesions. By day 28 p.i., the vast majority of animals have cleared the virus to levels below the detection ability of the plaque assay under non-stress conditions (Welsh 1990). Also by day 28 p.i., maximal inflammatory processes may have developed, while a differential development may be found earlier.

Results

Behavioral Measures of Illness

The direct comparison of the two timing schedules generally replicated the Behavioral effects found in Experiments 1 and 2, and extended the findings with other Behavioral measures as well.

Hindlimb impairment development occurred as expected based on the previous two experiments (see Figure 18). Infection alone resulted in hindlimb impairment that developed over time. Social disruption applied prior to infection exacerbated impairment, however stress applied concurrent with infection

reduced impairment. ANOVA confirmed a main effect for social disruption, F (2, 32) = 16.532, p < .0001, and significant interaction between Social Disruption X Day P.I., F (18,288) = 4.099, p < .0001. Post hoc means comparison indicated that at day 3 p.i. the animals that were stressed prior to infection had greater Hindlimb impairment than either the concurrently stressed animals or the

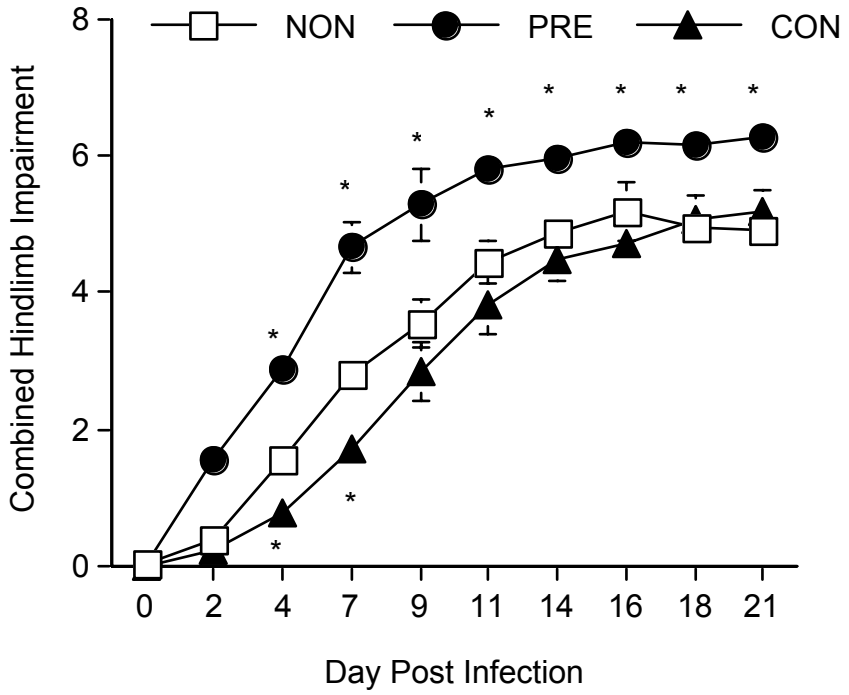

Figure 18. Hindlimb Impairment- Experiment 3.
Effect of social disruption applied either prior to infection (PRE) or concurrently with infection (CON) on the development of hindlimb impairment across time. Inoculation for all animals occurred at 9 p.m., on day 35 p.n. Controls were also infected at this time, but had no social disruption stress (NON).

controls; this difference was maintained throughout the experiment. In addition, by day 4 p.i., the concurrently stressed animals had less impairment than the control animals, and this remained so through day 9 p.i., when the difference between these two groups dissipated. This corresponds with the period of stress from day 0-7 p.i. for the concurrent condition.

Experiment 3 also incorporated scoring for encephalitis symptom development in addition to hindlimb impairment, as this scoring procedure has been applied to the previous restraint stress studies from the laboratory and others (McGavern, et al., 2001). Figure 19 depicts the sub-scales of ruffling of fur and hunching, signs of encephalitis. Ruffling developed due to infection in all animals. Ruffling is a measure of piloerection and failure to properly groom thought to be an important indicator of illness. Social disruption prior to infection slightly increased ruffling while concurrent social disruption slightly reduced ruffling. ANOVA revealed only a significant Social Disruption X Day P.I. effect, $F(18,297) = 1.629$, $p < .05$, while the main effect for social disruption was not found significant ($p > .05$). Post hoc analysis revealed more severe ruffling in animals exposed to social disruption prior to infection compared to the control and concurrent groups at day 4 p.i. Furthermore, at day 11 p.i., the concurrently stressed group was less ruffled than the other two groups.

Hunching developed at a similar rate in all the animals, but social disruption prior to infection had earlier onset compared to controls, and concurrently stressed animals had delayed onset of hunching. These effects were confirmed by ANOVA. A significant main effect for stress, $F(2,33) = 4.214$, $p < .05$, but no interaction with time developed ($p > .05$). Post hoc analysis revealed that the concurrently stressed group developed less hunching than the other two groups. The other two groups did not differ.

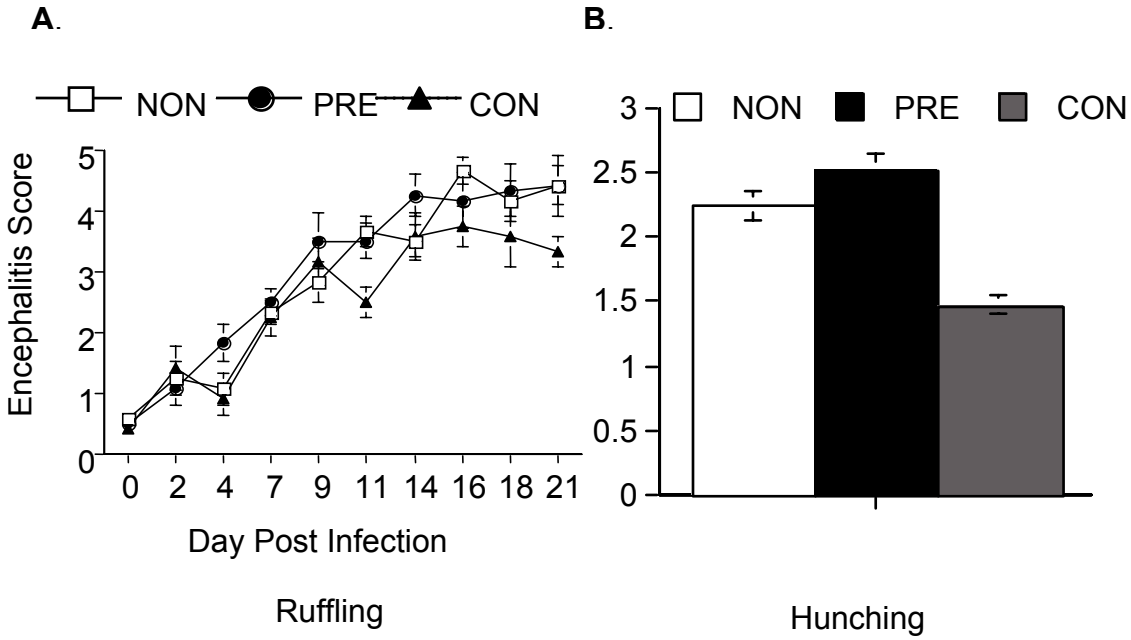

Figure 19. Encephalitis Subscales- Experiment 3.
Effect of social disruption on mean symptoms of ruffling (A) and hunching (B) as a function of social disruption +/- SEM. Ruffling developed across time as a function of social disruption, hunching developed as a function of social disruption, but did not change with time.

Grid hang latency is an objective measure of hindlimb strength. As expected infection alone reduced hang time during the course of infection. Social disruption prior to infection reduced this time even further, while concurrent social disruption did not effect hang time (Figure 20). Although the main effects and interaction terms failed to reach significance, a linear trend was found ($p < .05$), indicating that social disruption prior to infection resulted in the shortest hang times.

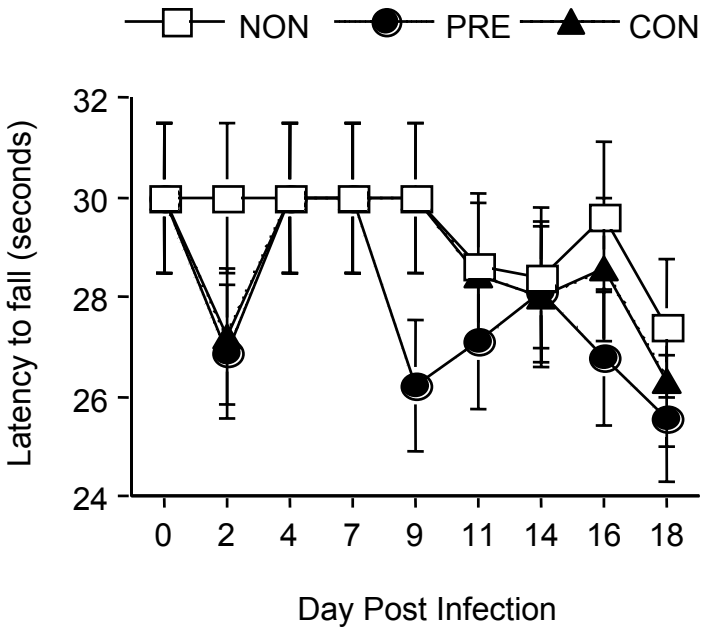

Figure 20. Grid Hang- Experiment 3.
Comparison of mean grid hang time +/- SEM as a function of socia disruption across time. Social disruption was applied either prior to infection (PRE), concurrent with infection (CON), or not at all (NON). For this experiment, the maximum required hang time was 30 seconds, after which the subject was removed.

Rotarod latencies as a function of social disruption across time are presented in Figure 21. Infection alone reduced time on the rotarod, F (2,4) = 3.673. $p < .01$, but social disruption did not alter infection-induced reductions, $p > .05$.

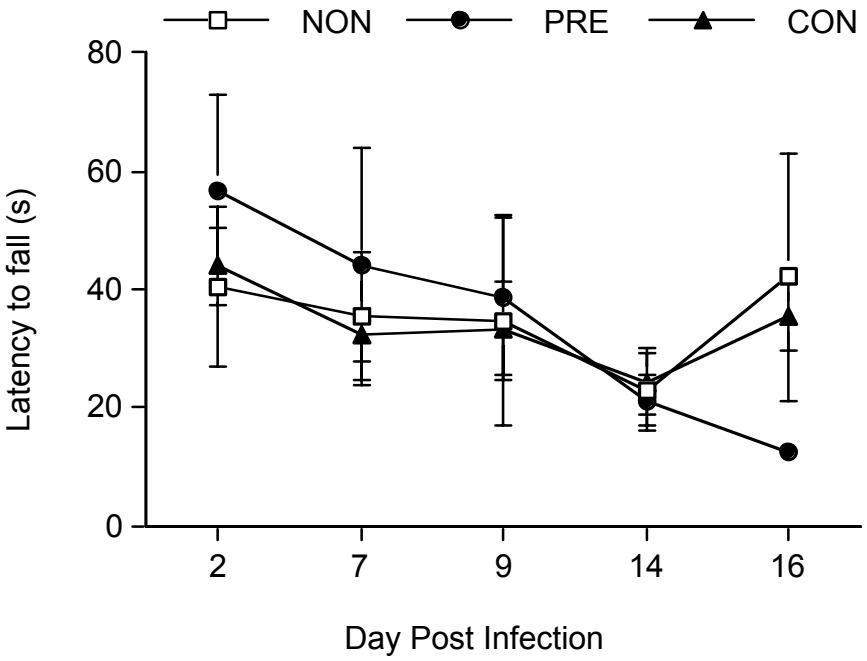

Figure 21. Rotarod- Experiment 3.
Effect of social disruption on the latency of time on rotarod (s) +/- SEM. All animals were infected and thus latencies were reduced across time. Maximum time allowed was 200 seconds.

Stride length on the footprint analysis at day 20 p.i. as a function of social disruption is shown in Figure 22. Social disruption applied prior to infection reduced stride length. ANOVA verified a main effect of social disruption, $F(2,32) = 5.475$, $p < .01$. Post hoc means comparisons revealed that stress applied prior to infection resulted in a decreased stride length.

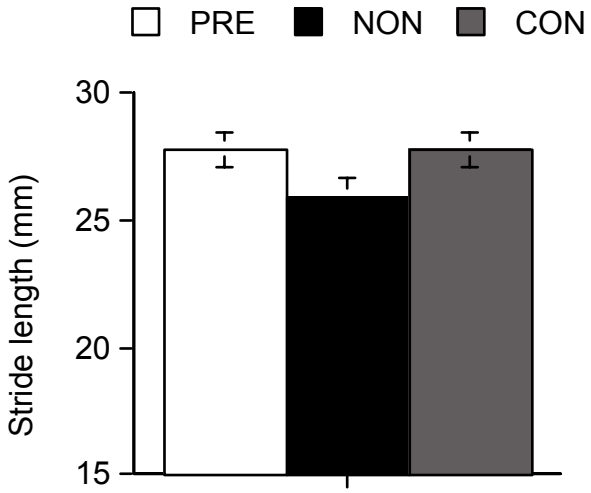

Figure 22. Footprint Stride Length- Experiment 3. Comparison of mean stride length +/- SEM at day 20 p.i., as a function of social disruption on the foot print assay. All animals were infected at day 0 p.i., and social disruption (SDR) was either applied prior to infection (PRE), or concurrent with infection (CON).

Weight gain as a function of time and social disruption is depicted in Figure 23. As found previously, social disruption applied prior to infection reduced normal weight gain. However, social disruption administered concurrent with infection failed to increase weight gain, as was found in Experiment 2. ANOVA verified a main effect of social disruption, F (2,32) = 4.068, $p < .05$, and a significant Social Disruption X Day P.I., F (16, 256) = 1.712, $p < .05$. Post hoc mean comparisons revealed that stress applied prior to infection resulted in reduced weight gain, while stress applied at the time of infection did not differ from the control condition.

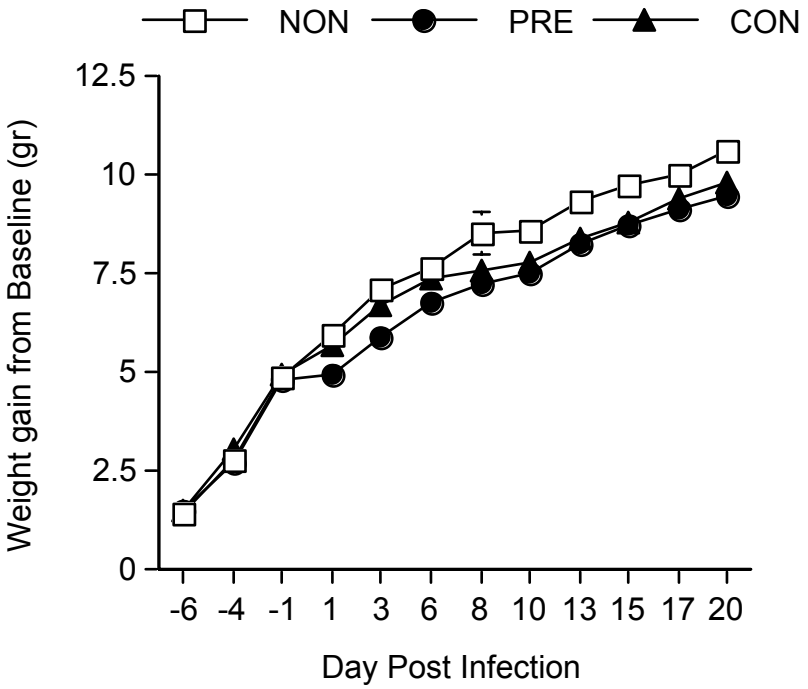

Figure 23. Body Weight Gain- Experiment 3.
Comparison of mean body weight gain (gr) across time as a function of social disruption +/- SEM. Social disruption was applied either prior to infection (PRE), concurrent with infection (CON), or not at all (NON).

Generally, the Behavioral measures of Hindlimb paralysis, encephalitis signs, grid hang latency, rotarod, and footprint all indicate that stress administered prior to infection resulted in a more severe disease course.

Physiological measures of disease course

Viral load and clearance are presented in Figure 24. The viral load and viral clearance were altered by social disruption only within the spinal cord. At day 7 p.i., an ANOVA revealed a significant main effect for social disruption $F(2,15) = 5.474$, $p < .05$, and a separate ANOVA demonstrated a significant main effect for social disruption at day 21 p.i., $F(2,15) = 3.993$, $p < .05$. An ANOVA

analysis of viral clearance also revealed a significant Social Disruption X Day P.I., F (2, 28) = 6.259, p < .01. Post hoc mean comparison showed that at day 7 p.i., animals exposed to social disruption prior to infection had the least amount of virus. Furthermore, post hoc mean comparisons demonstrated that both the control and concurrently socially disrupted groups significantly reduced viral load from day 7 p.i. to day 21 p.i., while the animals administered social disruption prior to infection showed no change in viral load.

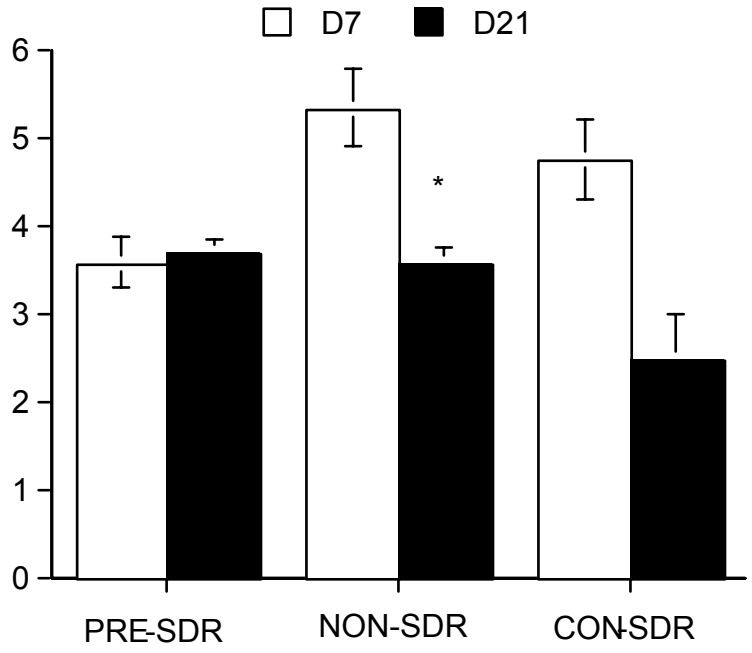

Figure 24. Viral Load and Clearance- Experiment 3.
Viral load and clearance in mean plaque forming units, +/- SEM as a function of social disruption (SDR) in spinal cord tissue. Social disruption was applied either prior to infection (PRE), concurrent with infection (CON), or not at all (NON). Tissue was collected at either day 7 p.i. or 21 p.i., all animals were infected.

Glucocorticoid resistance once again did not develop in any of the experimental or control conditions. These results are presented in Figure 25. ANOVA analysis revealed no differences due to social disruption ($p > .05$). Post hoc means comparisons of individual viability across corticosterone concentrations indicated that each did decrease significantly ($\underline{p} < .05$), indicating no glucocorticoid resistance developed.

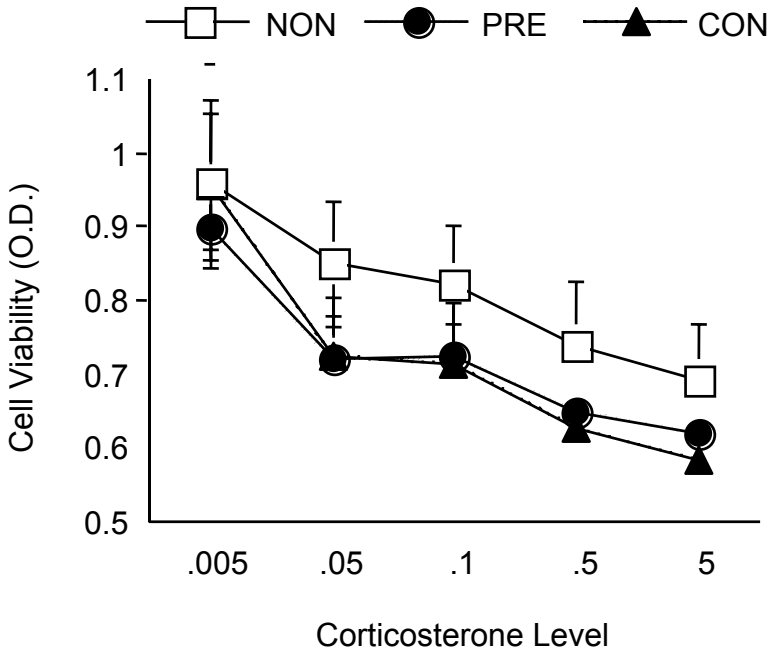

Figure 25. Glucocorticoid Resistance- Experiment 3.
Comparison of mean cell viability (O.D.) +/- SEM across increasing concentrations of corticosterone as a function of social disruption. All conditions failed to develop glucocorticoid resistance.

Figures 26 and 27 depict two series of histological analysis of the spinal cord and brain. Application of social disruption prior to infection resulted in an overall pattern of increased inflammation in spinal cord tissue. ANOVA confirmed this with separate analysis of each measure at each sacrifice date, demonstrating that a significant main effect for social disruption for meningitis, F (2,159) > 4.327, p < .05, and at day 21 p.i. for microgliosis, F (2,159) = 4.171, p < .05, and perivascular cuffing, F (2,159) = 3.002, p < .05. Post hoc means comparisons confirmed that social disruption applied prior to infection resulted in greater inflammation, at both sacrifice points. At day 7 p.i. both meningitis and microgliosis demonstrated greater inflammation in animals exposed to social disruption prior to infection compared to the control (non-stressed) condition, but not compared to the concurrently stressed animals or on perivascular cuffing (Figure 26). At day 21 p.i., inflammation was consistently increased in the social disruption prior to infection group compared to both control animals and concurrently stressed animals.

As with the viral clearance data, the brain tissue was less consistent than the spinal cord in the pattern of inflammation. Figure 27 presents the histological analysis of brain tissue as a function of social disruption. No differences were found to be significant within the brain, p > .05. However, the overall amount of inflammation in the brains was significantly less than in the spinal cord, p < .01. Post hoc means comparisons revealed that the spinal cord tissue has greater inflammation overall compared to brains, p < .05.

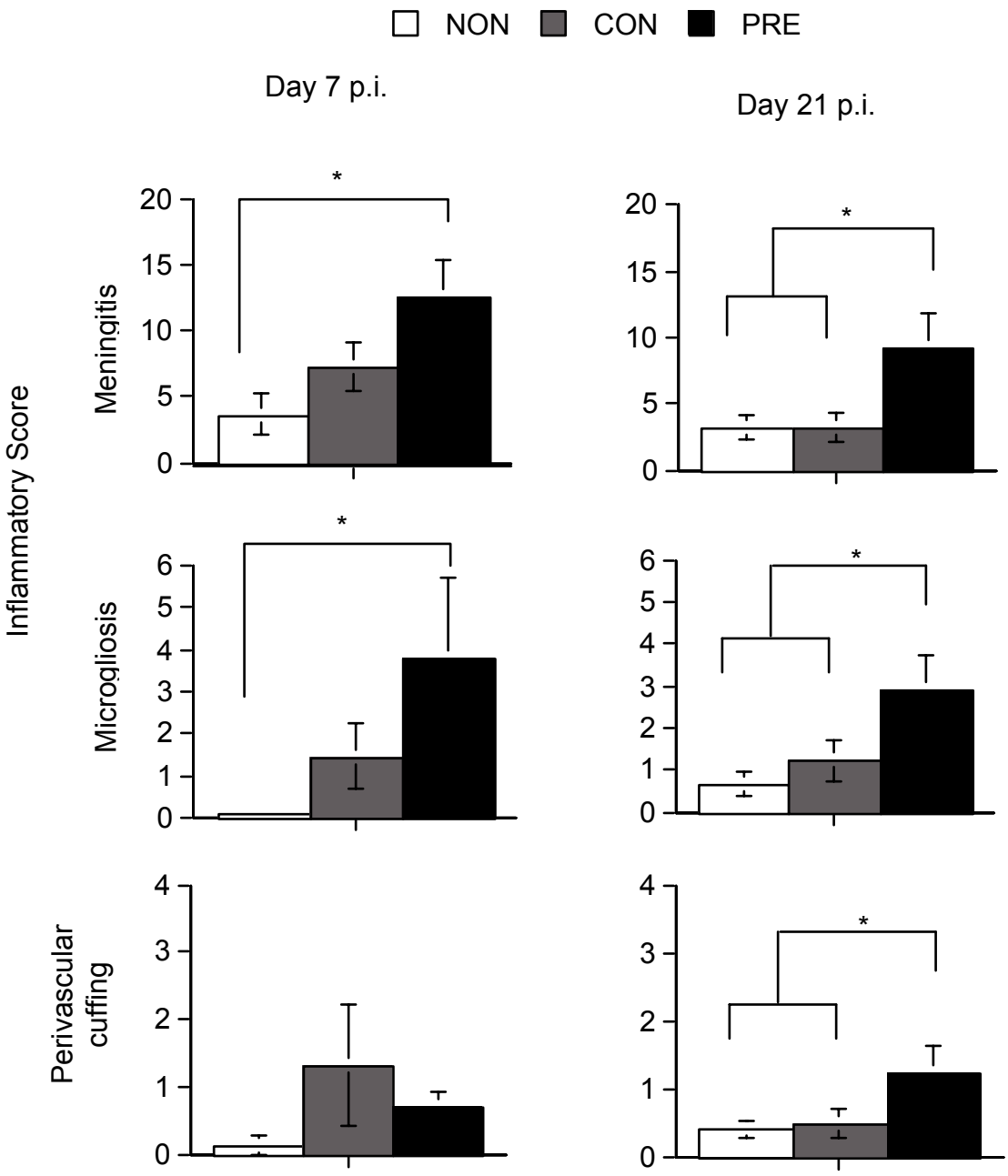

Figure 26. Spinal Cord Histology- Experiment 3.
Mean inflammatory score of both microgliosis and meningitis in spinal cord tissue as a function of social disruption (prior to infection, PRE, concurrent with infection, CON, and control, NON). All were infected.

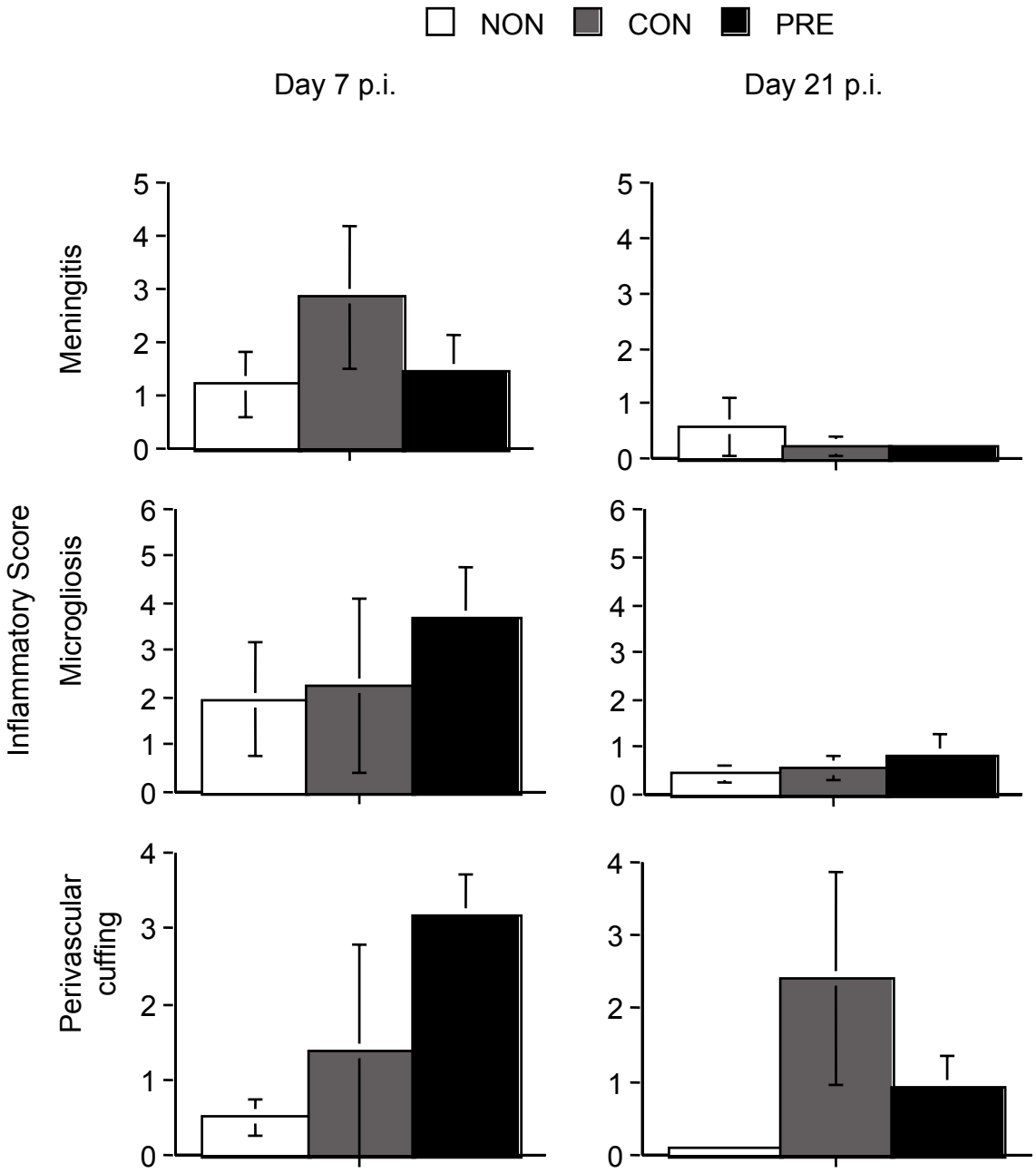

Figure 18. Brain Histology- Experiment 3.
Mean inflammatory score of microgliosis, meningitis, and perivascular cuffing in brain tissue as a function of social disruption (prior to infection, PRE; concurrent with infection, CON, and control, NON). All were infected.

Discussion

The present study demonstrated that social disruption applied prior to infection continued to result in a detrimental disease course outcome that may be associated with increased inflammation within the CNS. In addition, concurrent social disruption continued to reduce the severity of Theiler's virus infection. The Behavioral measures indicate that stress prior to infection altered disease course. Histologically, more severe Behavioral symptoms were associated with greater inflammation in the CNS ($r = .568$) in the animals exposed to social disruption prior to infection. More interesting perhaps is the viral clearance data. The other two groups (concurrently and non-stressed) had less inflammation overall, and greater variability, thus the histology and hindlimb impairment scores did not show as strong of a relationship. Viral loads at an early time point show that the groups with the least symptoms (non-stressed and concurrently stressed animals) had the larger amount of virus. However, by day 21 p.i., these two groups had cleared a significant amount of the virus, while the animals with the greater Behavioral signs of illness had not cleared any virus compared to the earlier time point. There appears to be a relationship between Behavioral signs of illness and ability to clear virus, and at least for the pre-stressed animals with histological inflammation as well.

GENERAL DISCUSSION

The present series of experiments were devised to investigate the complex relationship between stress and CNS inflammation as it relates to the development of neurodegenerative diseases. Overall, these experiments demonstrate that social disruption, is an effective moderator of Theiler's virus infection in both positive and negative directions. Many human studies examining stress and neurodegenerative diseases have found conflicting outcomes. Some have found deleterious outcomes associated with periods of stress (Franklin et al., 1988; Grant et al., 1989; Mohr et al., 2000; Motomura et al., 1998; Schwab and Zieper, 1965; Stip and Truelle, 1994; Warren et al., 1982; Warren et al., 1991). However, others have noted protective effects (Chelmicka-Schorr and Arnason, 1994; Nisipeanu and Korczyn, 1993). Finally some researchers have found that stress does not alter immune function in MS patients (Ackerman et al., 1996; Ackerman et al., 1998).

The current experiments elucidate one aspect of stress experiences that may explain these divergent findings- that of timing of stress in relation to disease course. Social disruption may be either deleterious, in the case of stress applied prior to infection, or advantageous, in the case of stress applied concurrent with infection. Recently it has been demonstrated that restraint stress also has an effect of timing in relation to immune challenge (Flint et al., 2001). However, restraint effects contrast with those observed in the current studies: restraint stress prior to sensitization led to decreased delayed type hypersensitivity responses when challenged, while application immediately prior to challenge led to an increased response. Although these two sets of data demonstrate contrasting outcomes, they both demonstrate the importance of timing when considering the stress-immune interaction. These two studies also address the importance of comparing qualitatively different stressors as well.

The timing of social disruption in relation to infection results in divergent Behavioral and physiological outcomes. Social disruption applied prior to

infection consistently results in greater Behavioral signs of illness, including hindlimb impairment, encephalitic signs, and stride length reduction. These Behavioral signs are associated with greater inflammation and a failure to reduce viral load across time. In contrast, social disruption applied concurrent with infection results in a reduction of Behavioral symptoms. In addition, inflammation is not significantly greater in concurrently stressed animals than in infected/ non-stressed controls. Finally, the concurrently stressed animals show the greatest reduction in viral load across time.

These experiments also emphasize the divergent impact of qualitatively different stressors on Theiler's virus infection. Prior studies examining the effects of restraint stress on Theiler's virus infection (Campbell et. al., 2001) can be compared to Experiments 2 and 3 because they used similar timing schedules. Campbell and colleagues (2001) found that restraint stress reduced inflammation but increased Behavioral signs of illness and mortality. In addition, viral loads were higher and clearance of virus was impaired. In contrast, concurrent social disruption increases inflammation non-significantly when comparing to infected/ controls. Finally, while early viral load is higher, the immuno-competency to clear the virus is superior to infected/ controls. These mice also show the least Behavioral signs of illness. Clearly these results indicate that these two stressors impact immune function in very different ways. Others have also found this to be true. Quan and colleagues (2000) directly compared restraint stress and social disruption when applied prior to immune challenge and found that social disruption was actually more detrimental to the immune challenge response. Sheridan and colleagues (2000) also found that a similar social stressor, social reorganization, lead to increased mortality compared to restraint stress in influenza infection.

Previous social disruption studies (Quan et al., 2001; Stark et al., 2002; Stark et al., 2001) have demonstrated that greater inflammation results when social disruption is applied prior to an immune challenge. The current studies

supported this observation. However, the pre-stressed animals also have reduced viral clearance capability. In addition, if these two measures (viral clearance and histological inflammation) are examined in the infected/ control animals in Experiment 3, the opposite pattern develops: the least amount of inflammation develops, but a significant reduction in virus occurs across time. Of even greater interest, perhaps, is the situation in the concurrently stressed animals. These mice have a moderate amount of inflammation that is not significantly higher than the infected/ controls. Despite this similarity, viral load reduction was significantly better in the concurrently stressed group. Some have noted that an optimal level of inflammation is important in many anti-viral immune responses (Salazar-Mather et al., 2002; Salazar-Mather et al., 1998). Taken together, these data indicate that an optimal level of inflammation may exist and at that level the immune response is most efficient. Higher or lower levels may impair immune function or even exacerbate the immune challenge.

Initial social disruption studies (Quan et al., 2001) also found that social disruption induced glucocorticoid resistance when applied prior to an immune challenge, while restraint stress did not. This was also true of social reorganization stress (Sheridan et al., 2000). Glucocorticoid resistance is thought to be associated with the greatly exacerbated inflammatory responses found in those studies. The current study replicated the increased inflammation in the animals exposed to social disruption prior to infection, but failed to find glucocorticoid resistance (in any mice exposed to social disruption either prior to or concurrent with infection). This may be due to several factors, including the extended immune challenge used here instead of the brief challenges used in the other laboratory. It may also be due to the age or strain of the mice used. The mice used in these experiments were younger (4-5 wk) compared to other social disruption studies (7-8 wk) at the time of social disruption. Additionally, the other social disruption studies utilized C57BL/6 mice, whereas the current studies used BALB/cJ mice. These disparities result from Theiler's virus

susceptibility limitations due to both strain and age. The mice used previously in social disruption experiments are not susceptible to Theiler's virus.

Although glucocorticoid resistance does not appear to be a mechanism in the Theiler's virus infection inflammatory process, other inflammatory mechanisms are indicated by the inflammation findings. Both stress and infection can induce pro-inflammatory cytokines leading to an increased inflammatory response (Maes, 2001; McGeer and McGeer, 1995; Stark et al., 2002). The most commonly found proinflammatory cytokines, TNF-α, IL-1 and IL-6, are all possible mediators of the effects found in these experiments and should be examined further.

Original conceptualizations of stress described a generalized phenomenon that occurred in a similar manner to multiple challenges (Selye, 1946). Mason (1971) first seriously challenged this conceptualization in a paper that described differential hormonal activation patterns for different types of stressors. Herman and colleagues (1997) have definitively found that different classes of stressors (processive or systemic) activate separate brain pathways. All stress is clearly not created equal! Many recent studies note that multiple variables associated with a stressor (chronicity, subject characteristics, etc) also impact how a stressor will moderate immune function. The present studies adds timing in relation to immune challenge to this list of variables.

REFERENCES

Ackerman, K.D., Martino, M., Heyman, R., Moyna, N.M., Rabin, B.S., 1996. Immunologic response to acute psychological stress in MS patients and controls. J. Neuroimmunol. 68, 85-94.

Ackerman, K.D., Martino, M., Heyman, R., Moyna, N.M., Rabin, B.S., 1998. Stressor-induced alteration of cytokine production in multiple sclerosis patients and controls. Psychosom. Med. 60, 484-491.

Ackerman, K.D., Rabin, B.S., Heyman, R., Baum, A., 2000. Stressful life events and disease activity in multiple sclerosis. Brain Behav.. Immun. 14, 77.

Adams, N., Lins, M.D., Blizard, D.A., 1987. Contrasting effects of social stress and foot-shock on acute cardiovascular response in salt-sensitive rats. Behav. Neural. Biol. 48, 368-381.

Andersson, P.B., Perry, V.H., Gordon, S., 1992. The acute inflammatory response to lipopolysaccharide in CNS parenchyma differs from that in other body tissues. Neuroscience 48, 169-186.

Aubert, C., Chamorro, M., Brahic, M., 1987. Identification of Theiler's virus infected cells in the central nervous system of the mouse during demyelinating disease. Microb. Pathog. 3, 319-326.

Avitsur, R., Stark, J.L., Dhabhar, F.S., Sheridan, J.F., 2002. Social stress alters splenocyte phenotype and function. J. Neuroimmunol. 132, 66-71.

Avitsur, R., Stark, J.L., Sheridan, J.F., 2001. Social stress induces glucocorticoid resistance in subordinate animals. Horm. Behav. 39, 247-257.

Bilbo, S.D., Dhabhar, F.S., Viswanathan, K., Saul, A., Yellon, S.M., Nelson, R.J., 2002a. Short day lengths augment stress-induced leukocyte trafficking and stress-induced enhancement of skin immune function. Proc. Natl. Acad. Sci. U. S. A. 99, 4067-4072.

Bilbo, S.D., Drazen, D.L., Quan, N., He, L., Nelson, R.J., 2002b. Short day lengths attenuate the symptoms of infection in Siberian hamsters. Proc. R. Soc. Lond. B. Biol. Sci. 269, 447-454.

Busciglio, J., Andersen, J.K., Schipper, H.M., Gilad, G.M., McCarty, R., Marzatico, F., Toussaint, O., 1998. Stress, aging, and neurodegenerative disorders. Molecular mechanisms. Ann. N. Y. Acad. Sci. 851, 429-443.

Campbell, T., Meagher, M.W., Sieve, A., Scott, B., Storts, R., Welsh, T.H., Welsh, C.J., 2001. The effects of restraint stress on the neuropathogenesis of Theiler's virus infection: I. Acute disease. Brain Behav. Immun. 15, 235-254.

Chelmicka-Schorr, E., Arnason, B.G., 1994. Nervous system-immune system interactions and their role in multiple sclerosis. Ann. Neurol. 36 Suppl., S29-32.

Cirulli, F., Pistillo, L., de Acetis, L., Alleva, E., Aloe, L., 1998. Increased number of mast cells in the central nervous system of adult male mice following chronic subordination stress. Brain Behav. Immun. 12, 123-133.

de Vries, H.E., Kuiper, J., de Boer, A.G., Van Berkel, T.J., Breimer, D.D., 1997. The blood-brain barrier in neuroinflammatory diseases. Pharmacol. Rev. 49, 143-155.

DeRijk, R., Sternberg, E.M., 1997. Corticosteroid resistance and disease. Ann. Med. 29, 79-82.

Dhabhar, F.S., 1998. Stress-induced enhancement of cell-mediated immunity. Ann. N. Y. Acad. Sci. 840, 359-372.

Dhabhar, F.S., McEwen, B.S., 1996. Stress-induced enhancement of antigen-specific cell-mediated immunity. J. Immunol. 156, 2608-2615.

Dhabhar, F.S., McEwen, B.S., 1997. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. Brain Behav. Immun. 11, 286-306.

Dhabhar, F.S., McEwen, B.S., 1999. Enhancing versus suppressive effects of stress hormones on skin immune function. Proc. Natl. Acad. Sci. U. S. A. 96, 1059-1064.

Dhabhar, F.S., Miller, A.H., Stein, M., McEwen, B.S., Spencer, R.L., 1994. Diurnal and acute stress-induced changes in distribution of peripheral blood leukocyte subpopulations. Brain Behav. Immun. 8, 66-79.

Esposito, P., Gheorghe, D., Kandere, K., Pang, X., Connolly, R., Jacobson, S., Theoharides, T.C., 2001. Acute stress increases permeability of the blood-brain-barrier through activation of brain mast cells. Brain Res. 888, 117-127.

Flint, M.S., Valosen, J.M., Johnson, E.A., Miller, D.B., Tinkle, S.S., 2001. Restraint stress applied prior to chemical sensitization modulates the development of allergic contact dermatitis differently than restraint prior to challenge. J. Neuroimmunol. 113, 72-80.

Floyd, R.A., 1999. Neuroinflammatory processes are important in neurodegenerative diseases: an hypothesis to explain the increased formation of reactive oxygen and nitrogen species as major factors involved in neurodegenerative disease development. Free Radii. Biol. Med. 26, 1346-1355.

Franklin, G.M., Heaton, R.K., Nelson, L.M., Filly, C.M., Seibert, C., 1988. Correlation of neuropsychological and MRI findings in chronic/progressive multiple sclerosis. Neurology 38, 1826-1829.

Fujinami, R.S., Zurbriggen, A., 1989. Is Theiler's murine encephalomyelitis virus infection of mice an autoimmune disease? Apmis 97, 1-8.

Gonzalez-Scarano, F., Baltuch, G., 1999. Microglia as mediators of inflammatory and degenerative diseases. Ann. Rev. Neurosci. 22, 219-240.

Grant, I., Brown, G.W., Harris, T., McDonald, W.I., Patterson, T., Trimble, M.R., 1989. Severely threatening events and marked life difficulties preceding onset or exacerbation of multiple sclerosis. J. Neurol. Neurosurg. Psychiatry 52, 8-13.

Herman, J.P., Cullinan, W.E., 1997. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci. 20, 78-84.

Kerr, L., Drummond, L., Zaharia, M., Clelford, J., Anisman, H., 1996. Stressor-induced alterations of the splenic plaque-forming cell response: strain differences and modification by propranolol. Pharmacol. Biochem. Behav. 53, 235-241.

Lassmann, H., 1991. Inflammation and the blood-brain barrier. Brain Pathol. 1, 88.

Lassmann, H., Zimprich, F., Rossler, K., Vass, K., 1991. Inflammation in the nervous system. Basic mechanisms and immunological concepts. Rev. Neurol.(Paris) 147, 763-781.

Lipton, H.L., 1975. Theiler's virus infection in mice: an unusual biphasic disease process leading to demyelination. Infect. Immun. 11, 1147-1155.

Maes, M., 2001. Psychological stress and the inflammatory response system. Clin. Sci. (Lond) 101, 193-194.

Maes, M., Song, C., Lin, A., De Jongh, R., Van Gastel, A., Kenis, G., Bosmans, E., De Meester, I., Benoy, I., Neels, H., Demedts, P., Janca, A., Scharpe, S., Smith, R.S., 1998. The effects of psychological stress on humans: increased production of pro-inflammatory cytokines and a Th1-like response in stress-induced anxiety. Cytokine 10, 313-318.

Mason, J.W., 1971. A re-evaluation of the concept of "non-specificity" in stress theory. J. Psychiatr. Res. 8, 323-333.

Matyszak, M.K., Perry, V.H., 1995. Demyelination in the central nervous system following a delayed-type hypersensitivity response to bacillus Calmette-Guerin. Neuroscience 64, 967-977.

Matyszak, M.K., Townsend, M.J., Perry, V.H., 1997. Ultrastructural studies of an immune-mediated inflammatory response in the CNS parenchyma directed against a non-CNS antigen. Neuroscience 78, 549-560.

McGavern, D.B., Zoecklein, L., Drescher, K.M., Rodriguez, M., 1999. Quantitative assessment of neurologic deficits in a chronic progressive murine model of CNS demyelination. Exp. Neurol. 158, 171-181.

McGavern, D.B., Zoecklein, L., Sathornsumetee, S., Rodriguez, M., 2000. Assessment of hindlimb gait as a powerful indicator of axonal loss in a murine model of progressive CNS demyelination. Brain Res. 877, 396-400.

McGeer, P.L., McGeer, E.G., 1995. The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Res. Brain Res. Rev. 21, 195-218.

Mohr, D.C., Goodkin, D.E., Bacchetti, P., Boudewyn, A.C., Huang, L., Marrietta, P., Cheuk, W., Dee, B., 2000. Psychological stress and the subsequent appearance of new brain MRI lesions in MS. Neurology 55, 55-61.

Motomura, N., Ohkubo, F., Tomota, Y., Akagi, H., Asano, A., Seo, T., 1998. Premorbid psychosocial Behavior in demented patients. Int. J. Neurosci. 95, 167-172.

Nguyen, M.D., Julien, J.P., Rivest, S., 2002. Innate immunity: the missing link in neuroprotection and neurodegeneration? Nat Rev Neurosci 3, 216-227.

Nisipeanu, P., Korczyn, A.D., 1993. Psychological stress as risk factor for exacerbations in multiple sclerosis. Neurology 43, 1311-1312.

Paik, I.H., Toh, K.Y., Lee, C., Kim, J.J., Lee, S.J., 2000. Psychological stress may induce increased humoral and decreased cellular immunity. Behav. Med. 26, 139-141.

Palanza, P., Gioiosa, L., Parmigiani, S., 2001. Social stress in mice: gender differences and effects of estrous cycle and social dominance. Physiol. Behav. 73, 411-420.

Quan, N., Avitsur, R., Stark, J.L., He, L., Shah, M., Caligiuri, M., Padgett, D.A., Marucha, P.T., Sheridan, J.F., 2001. Social stress increases the susceptibility to endotoxic shock. J. Neuroimmunol. 115, 36-45.

Rosch, P.J., 1979. Stress and illness. J. A. M. A. 242, 417-418.

Salazar-Mather, T.P., Lewis, C.A., Biron, C.A., 2002. Type I interferons regulate inflammatory cell trafficking and macrophage inflammatory protein 1alpha delivery to the liver. J. Clin. Invest. 110, 321-330.

Salazar-Mather, T.P., Orange, J.S., Biron, C.A., 1998. Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1alpha (MIP-1alpha)-dependent pathways. J. Exp. Med. 187, 1-14.

Schwab, R.S., Zieper, I., 1965. Effects of mood, motivation, stress and alertness on the performance in Parkinson's disease. Psychiatr. Neurol. (Basel) 150, 345-357.

Selye, H., 1946. The general adaptation syndrome and the disease of adaption. J. Clin. Endocrinol. 6, 117-230.

Sheridan, J.F., Stark, J.L., Avitsur, R., Padgett, D.A., 2000. Social disruption, immunity, and susceptibility to viral infection. Role of glucocorticoid insensitivity and NGF. Ann. N. Y. Acad. Sci. 917, 894-905.

Sibley, W.A., 1997. Risk factors in multiple sclerosis. In: Tortellotte, W.W. (Ed.), Multiple Sclerosis: Clinical and Pathogenetic basis, Chapman & Hall, London, pp. 141-148.

Stark, J.L., Avitsur, R., Hunzeker, J., Padgett, D.A., Sheridan, J.F., 2002. Interleukin-6 and the development of social disruption-induced glucocorticoid resistance. J. Neuroimmunol. 124, 9-15.

Stark, J.L., Avitsur, R., Padgett, D.A., Campbell, K.A., Beck, F.M., Sheridan, J.F., 2001. Social stress induces glucocorticoid resistance in macrophages. Am. J. Physiol. Regul. Integr. Comp. Physiol. 280, R1799-1805.

Sternberg, E.M., 2001. Neuroendocrine regulation of autoimmune/inflammatory disease. J. Endocrinol. 169, 429-435.

Stip, E., Truelle, J.L., 1994. Organic personality syndrome in multiple sclerosis and effect of stress on recurrent attacks. Can. J. Psychiatry 39, 27-33.

Theiler, M., 1934. Spontaneous encephalomyelitis of mice- a new virus disease. Science 80, 122.

Warren, S., Greenhill, S., Warren, K.G., 1982. Emotional stress and the development of multiple sclerosis: case-control evidence of a relationship. J. Chronic Dis. 35, 821-831.

Warren, S., Warren, K.G., Cockerill, R., 1991. Emotional stress and coping in multiple sclerosis (MS) exacerbations. J. Psychosom. Res. 35, 37-47.

Welsh, C.J., Tonks, P., Borrow, P., Nash, A.A., 1990. Theiler's virus: an experimental model of virus-induced demyelination. Autoimmunity 6, 105-112.

Zheng, C., Feinstein, P., Bozza, T., Rodriguez, I., Mombaerts, P., 2000. Peripheral olfactory projections are differentially affected in mice deficient in a cyclic nucleotide-gated channel subunit. Neuron 26, 81-91.

APPENDIX A

Hindlimb Impairment Scoring

Score	Description
0	Healthy, no weakness, able to cling well, body tight to grid
1	Slight weakness in grip, uses too much toe, still tight against grid
2	Clear weakness in grip, uses tips of toes, bats legs to reposition, dangles down from grid when inverted
3	Clear weakness and slight paralysis, bats, cannot reposition well, may drag limb but has full usage, does not move as much
4	Clear weakness and moderate paralysis, may drag limb, but able to use, clinging is lost
5	Complete paralysis, limb does not move, limb is usually tight against body

Symptoms to note for numerical score:

Weakness (slight to very)

Paralysis (slight, moderate, complete)

Dragging limb (side or back)

Cling (yes or no)

Movement (yes or no)

Amount of motion (normal, slow)

Technique:

Place mouse on a standard mouse cage floor grid, invert grid so that the mouse is hanging. Watch for use of toes, how tight against the grid the mouse stays, leg batting and repositioning toes onto the grid when moving or grip is lost. Also note if leg moves or not in later stages.

APPENDIX B

Encephalitis Scoring

Score	Description
0	healthy (good grooming, active, bright eyes)
.5	slightly ruffled fur
1	clearly ruffled fur and slight hunching
2	Clear hunching, lethargic or vocalize to touch
3	Very hunched, lethargic, sunken eyes, unresponsive to touch
4	Moribund

Symptoms noted for numerical score

Grooming (good, incomplete, bad)

Ruffled fur (slight, moderate, very)

Hunching (slight, very)

Lethargic (slight, very)

Sunken eyes (right, left, or both)

Vocalize to touch/responds to touch

Loose bowel/diarrhea

Also note:

Lost fur

Feel cold

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Publications (in preparation):

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Published Abstracts:

Johnson, R. R., Welsh, T., Tiffany-Castiglioni, E., Welsh, C.J.R., and Meagher, M.W. (2001). Social disruption stress increases severity of paralysis in BALB/cJ mice infected with Theiler's virus. Society for Neuroscience Abstracts, 27, 843.5.

Johnson, R. R., Welsh, T. H., Husband, L. A., Welsh, C.J.R., and Meagher, M. W. (2002). The timing of social stress alters the course of Theiler's virus infection. Brain, Behavior and Immunity, 16 (2), 193-194.

Johnson, R.R., Welsh, T.H. Storts, R., Welsh, C.J.R., and Meagher, M.W. (2002). Timing of social stress alters Theiler's virus induced CNS inflammation. Society for Neuroscience Abstracts, 28. 576.12.